Annex

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ERRATA

Article by Broadhurst, "Environmental studies of streptococci with special reference to the fermentative reactions," Vol. 17, No. 2, pp. 277-330.

(*a*) In Table 15 (opposite p. 318) the following should be added to the footnote on litmus milk: — A ═ slightly alkaline; Alk ═ definitely alkaline (never strongly so, however).

(*b*) In Table 17 (following p. 323) under "Percentage Fermenting" and "Sections 1-4" the first two lines of figures, referring to Andrewes and Horder's strains, should be transposed.

(*c*) In Table 17 a footnote should be added as follows: "Most of Houston's strains evidently belong in the raffinose section."

Article by Mathers, "The varieties of pneumococci causing labor pneumonia with especial reference to their biologic differences," Vol. 17, No. 3, p. 515, line 7, should read "1-2 c.c." instead of "12 c.c."

THE USE OF BRILLIANT GREEN FOR THE ISOLATION OF TYPHOID AND PARATYPHOID BACILLI FROM FECES [*]

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AND

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While investigating the effect of various aniline dyes on the growth of bacteria[1] we found that brilliant green, aniline green, solid green, smaragd green, and china green, in appropriate dilutions, consistently restrained the growth of the gram-positive bacteria but varied in their action on the gram-negative bacteria. The most important variations were among the members of the colon-typhoid group. The growth of the typhoid bacillus was restrained but slightly, the paratyphoid-enteritidis types grew abundantly, whereas many of the colon group grew feebly or not at all. Many aerogenes types, as well as Bacillus proteus and Bacillus pyocyaneus, were unaffected, even when the dye was present in low dilutions. The concentrations at which this differential action was evident varied with the individual dyes. No one dye seemed more selective than another, but brilliant green exhibited the selective action at higher dilutions.

On the basis of these results, we suggested the use of brilliant-green broth for the enrichment of typhoid and paratyphoid bacilli in feces. Browning, Gilmour, and Mackie[2] suggested the same procedure for the enrichment of the typhoid bacillus, and Torrey,[3] for the enrichment of the paratyphoid-enteritidis types. The following is a report of the results of the application of this method and of further investigations of the action of brilliant green in fluid and solid media.

It had been found that the addition of feces to brilliant-green broth reduced the activity of the dye. It was necessary, therefore, in order to obtain the point of optimal differential action, either to use graded dilutions of the dye or to vary the amount of feces.

The method (Mr. Lawrence A. Kohn assisted us in carrying out the technical part of this work) employed in examining stools for typhoid bacilli was to add 0.1 c.c. of a moderately heavy suspension of feces to each of 3 tubes containing 10 c.c. of 1% glucose extract broth, neutral to phenolphthalein, to which brilliant green had been added to give final dilutions of 1 : 300,000, 1 : 400,000, and 1 : 500,000. After 15 to 18 hours' incubation, a loop from each tube was streaked on Endo plates, and these examined for typhoid colonies after incubation. Endo plates (Kendall's modification) were also inoculated directly from the suspension of feces used for the dye-broth tubes. The results obtained with this method in routine examinations for typhoid are given in Table 1.

TABLE 1

RESULTS OF EXAMINATION FOR TYPHOID BACILLI IN FECES IN BRILLIANT-GREEN BROTH

Number of Stools Examined	Result, Endo, Direct Inoculation	Result, Endo, Inoculation from Green-Dye Broth
134...	Negative	Negative
26...	Positive	Positive
19...	Positive	Negative
4...	Negative	Positive
183		

In the case of the stools giving positive results with both methods, there was, in some instances, a relative increase of the typhoid bacilli; in others there was a decrease. When stools were positive on direct plating and negative from the dye broth, the loss of the typhoid bacilli was due to the over-growth of dye-resistant organisms, especially the mucoid aerogenes types, and, less frequently, the paratyphoid-like intermediates or members of the proteus and pyocyaneus

[2] Jour. Hyg., 1913, 13, p. 335.
[3] Jour. Infect. Dis., 1913, 13, p. 263.

groups. The following experiment shows how easily such overgrowth may occur.

To 5 c.c. of a boiled suspension of feces were added 1 c.c. of a broth culture of typhoid and an equal volume of a broth culture of Bacillus [lactis] aerogenes. One-half cubic centimeter of this mixture was added to glucose broth containing 1 : 400,000 of brilliant green. This was plated, incubated, and plated at intervals, to determine the proportion between the two types. Plated immediately, B. aerogenes was to B. typhosus as 5 to 1; after 3 hours, as 9 to 1; and after 24 hours, as 300 to 1.

The work showed that a large proportion of the fecal flora could be suppressed. If this suppression could be obtained by a shorter exposure to the action of the dye, the subsequent loss of the typhoid bacillus by overgrowth of dye-resistant types might be avoided. In this way, the number of typhoid bacilli would be relatively increased altho an actual enrichment due to multiplication would not occur.

Small amounts of suspensions of carrier stools (supplied to us by Dr. Anna M. Agnew of Long Island State Hospital) were added to suspensions of normal stools (to reduce the proportion of typhoid bacilli), and the resultant mixtures used for the following tests. After direct Endo plates had been made, 2 c.c. of the mixture were added to 4 c.c. of broth containing the dye. From this mixture, plates were made immediately and after 1, 3, and 18 hours, respectively. In some instances 2 dilutions of the dye, 1 : 100,000 and 1 : 300,000, were employed. No appreciable difference was noted. The results, with the use of a dilution of 1 : 300,000, are given in Table 2.

TABLE   2
Results of Examination for Typhoid Bacilli in Feces After Short Exposure to
Brilliant-Green Dye

Number of Specimens	Direct Plates	Plates Made from the Dye Broth at Intervals			
		Immediately	After 1 Hr.	After 3 Hr.	After 18 Hr.
1	Negative	Positive	Positive	Negative	Negative
1	Negative	Negative	Positive	Negative	Negative
1	Negative	Negative	Positive	Positive	Negative
1	Positive	Negative	Positive	Negative	Negative
7	Positive	Positive	Positive	Positive	Positive
3	Positive	Positive	Positive	Positive	Negative
Total	11 Positive	11 Positive	14 Positive	11 Positive	7 Positive
14	3 Negative	3 Negative	0 Negative	3 Negative	7 Negative
Average percentage of typhoid on plates	50%	60%	63%	45%	2%

The shorter exposure to the action of the dye gave a greater increase of positive results over those in direct plating than had been obtained with the previous attempts at enrichment by longer periods

of incubation. The growth on the plates showed, however, that this short exposure is not sufficient to prevent the growth of many of the dye-sensitive types present in the stool when they are subsequently transferred to a favorable medium.

The results with both methods were, on the whole, less satisfactory than had been anticipated. If a dye agar could be devised for direct plating, the suppression of the susceptible types would be more complete than when exposed for a short period to the action of the dye and then transferred to a favorable medium. Various combinations were tried but unsuccessfully, because the typhoid colonies were not sufficiently characteristic to allow of selection when other fecal types developed. It was thought at this time that an indicator was essential to differentiate the lactose-fermenting types. Many were tried but did not give sharp enough differences. The Endo indicator could not be used as the sodium sulfite reduces the activity of the brilliant green. The Andrade indicator, which consists of 100 c.c. of a 0.5% solution of acid fuchsin decolorized by the addition of 16 c.c. of a normal solution of sodium hydrate, was found to answer our purpose. The color of this indicator is restored by acids.

After many trials a medium of the following constitution was found to be satisfactory:

Liebig's extract of beef....................	3	grams
Witte's peptone............................	10	grams
Salt	5	grams
Agar	15	grams
Water	1,000	c.c.

Dissolve in the autoclave. The final reaction must be set to the Andrade indicator. This can be done at the time of preparation, or, as we have found more convenient, the agar can be rendered slightly alkaline to litmus and the final reaction set when used. If the reaction is to be set at once, 100 c.c. are placed in a bottle, 1 c.c. of the indicator added, and the whole titrated to a distinct red color, which will disappear on cooling. Time must elapse between the additions of acid or alkali, as the indicator adjusts itself slowly. The indicator is then added to the remainder of the agar, the reaction adjusted and finished in the ordinary manner. In the method we have employed, because of its greater convenience, the agar is rendered slightly alkaline to litmus, bottled in 100 c.c. amounts, and autoclaved. After determining the acid necessary by testing one bottle, this amount is added to the other bottles as used. This has the advantage that the reaction can be set more accurately in the clear agar and if the first bottle does not give completely satisfactory results, the amount can be varied. After addition of the acid the reaction to phenolphthalein (hot titration) is 0.6% to 0.7% acid.

In either case, the indicator should be added and the reaction adjusted before the sugars and dye are introduced. To the melted agar, just before use, are added 1% of lactose and 0.1% of glucose—sterile 25% solutions of

the sugars being used—and, finally, the appropriate amount of a 0.1% solution of brilliant green. Each 100 c.c. will suffice for 6 plates, as the layer of agar must be relatively thick else the characteristic appearance of the typhoid colony is not as marked. The plates are allowed to stand open until the agar has solidified and then are covered, preferably with porous covers, to absorb the moisture. Inoculation is the same as with Endo plates.

On this medium, many of the fecal types are restrained, whereas the typhoid bacillus produces large colonies which are very characteristic. Viewed through the plate against a dark background, with the light passing obliquely through the agar, they have a peculiar snow-flake appearance. Seen by artificial light and a hand lens, under the same conditions, they have the appearance of a coarse wool fabric. They vary slightly in distinctiveness; as a rule, the larger colonies are the more typical and show best where the plates develop a moderate number of colonies. Other fecal types may develop typhoid-like colonies, but this similarity is usually no more troublesome than with Endo or other media.

To test this medium under the most natural conditions obtainable, we added to normal stools small traces of carrier stools. In this way, stools were made containing very few typhoid bacilli. The following results were obtained when suspensions of these mixtures were plated on Endo and on the green-dye agar:

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Total examined............130
Endo agar................. 71 negative; 59, or 45%, positive.
Green-dye agar  (0.2 c.c. of
    a 0.1% solution of dye to
    100 c.c. of agar)....... 24 negative; 106, or 81%, positive.
Increased positive results.. 36%.
```

Of the 71 negative on Endo, 47 (or 67%) were positive on the green-dye agar. None was positive on Endo when negative on the dye agar.

Fifty-seven samples were also added to green-dye broth as in the preceding experiments, and, after one-half hour, plated on Endo. Of these, 8 were positive, altho negative on the direct Endo plates, but 6 were negative which on the direct Endo plates had been positive. Twenty-two were negative which had been positive on the direct green-dye plates. None was positive which on the direct green-dye plates had been negative.

Sixty-six samples were enriched in the same way, but plated on green-dye agar instead of Endo. Three were negative which had been positive on the direct green-dye plates and 3 were positive which

on the direct green-dye plates had been negative—as a result, in two instances, of the fact that the direct plates were overseeded.

At the same time 0.1 c.c. of the suspension was inoculated into 10 c.c. of glucose broth containing 1 : 300,000 of the dye, and after 18 hours' incubation plated on both Endo and green-dye agar. Forty-seven samples were tested. Six in both cases gave positive results which on the direct Endo plates had been negative, but 30 were negative which on the direct green-dye plates had been positive. In only one instance was the sample positive which on the direct green-dye plates, as well as on the Endo plates, had been negative.

While the comparative examinations described were being made, it was noted that with certain stools the number of colonies developing on the green-dye agar was only slightly less than on the Endo agar. Considerable difficulty was encountered in some instances because of the similarity of some of the colonies to those of the typhoid bacillus. These colonies were fished and found to be either intermediate types, slow lactose fermenters, or incompletely restrained colon types. It appeared to us that we might exclude these organisms with stronger dilutions of the dye. Altho the number of typhoid colonies was decreased, they should be more easily found, were the typhoid-like type not present. Comparative tests showed that the concentration of the dye could not be carried much farther than 1 : 300,000, else the typhoid bacillus might not develop. From these tests it was decided to prepare the agar as before, but to use 2 series : one to contain 0.2 c.c., the other 0.3 c.c. of a 0.1% solution of the dye to 100 c.c. of the agar. In this series of comparative tests, Conradi's brilliant-green agar, of which we shall speak later, was also included. The material used for inoculation was a mixture of carrier and normal stools. The results are given in the following table :

TABLE 3

RESULTS OF EXAMINATION FOR TYPHOID BACILLI IN FECES EXPOSED TO DIFFERENT CONCENTRA-
TIONS OF BRILLIANT GREEN

Result	Endo	Amount of 0.1% Dye Solution for 100 c.c. of Agar		Conradi
		0.2 c.c.	0.3 c.c.	
Negative	18	9	3	14
Positive	10	19	25	14
Average total of colonies developing	1,250	230	70	40
Average number of typhoid colonies	20	40	25	10

In one instance the agar containing 0.2 c.c. of the dye was positive, whereas the agar containing 0.3 c.c. was negative, because of almost complete inhibition of growth. In no instance were the Endo or the Conradi plates positive when our green-dye agar was negative. The figures given of the number of colonies developing on the different media are only roughly comparative, as more material was inoculated on the media containing the dye. They serve, however, to give an idea of the amount of restraint of the ordinary fecal types and the relative enrichment of the typhoid bacilli.

About this time a small, localized epidemic of typhoid occurring, we received 9 stools for diagnosis. Of these, none gave positive results on Endo medium, 4 were positive on the green-dye agar containing 0.2 c.c. of dye, and 5 were positive on the agar containing 0.3 c.c. of the dye. These natural stools acted as a control on the results obtained with normal stools inoculated with carrier stools.

The tests in this series were severe, as the stools were especially rich in dye-resistant types. When typhoid-like colonies developed on the agar containing less dye, they were in great part excluded on the agar containing more dye.

Table 4 gives the results of the routine examination of feces, chiefly from convalescents prior to discharge. This includes the specimens mentioned.

TABLE 4

Results of Routine Examination for Typhoid Bacilli in Feces

Number Examined	Endo	Green-Dye Agar	
		0.2 c.c.	0.3 c.c.
631	65 positive	89 positive 3 positive 7 negative	89 positive 3 negative 7 positive
		Total positive 99	

In two instances the typhoid bacilli in the stools were unusually sensitive to the dye, as shown by the development of small to fine colonies. In spite of this, one of the samples was positive on the dye plates only.

In the use of the green-dye agar as described, many points have arisen as to the importance of the different constituents, which have been made clear by a number of comparative tests. We can best show these points by discussing each.

The concentration of the agar cannot be varied from that given without finding a new optimal concentration of the dye. Thus, if the dye be used with 3% agar, there is a marked decrease in its action.

The concentration of the peptone and meat extract has been found satisfactory, and no variations have been tried. By analogy with other observations, we are certain that any increase in the concentration will be accompanied by a decrease in the activity of the dye.

The trace of glucose was added because we had previously found that this amount of sugar would markedly increase the size of cholera colonies. As this amount of glucose is employed in the Russell medium, we did not anticipate that there would be sufficient acid produced by surface colonies to affect the indicator. The presence of this trace of glucose is the fundamental factor in the success of our medium. The typhoid colony is usually larger and more important; its distinctiveness is increased. Large colonies are desirable, as they furnish ample material for macroscopic slide agglutinations, which allow a rapid survey of many colonies. When the reaction of the medium is set close to the neutral point of the indicator, the use of glucose is followed by a slight tinging of the colony, which, however, greatly increases its characteristicity. Comparative tests without glucose have given very poor results because of the lessened distinctiveness of the typhoid colony.

The indicator has been tried in different concentrations in the hope of accentuating the lactose-fermenting types. On the whole, this has made no appreciable difference and we have decided to employ 1%.

The lactose was added to differentiate the lactose-fermenting types. As many of them are inhibited, this is not so important as we at first considered it. Furthermore, most of the lactose-fermenting organisms which develop are either distinctive in their colony morphology (as the aerogenes types), or produce insufficient acid to affect the indicator. Similar results are noted in the use of the Endo agar, in which many colorless or nearly colorless colonies may develop, which, on isolation, are found to be lactose fermenters. On this account it might seem that the lactose and the indicator could be omitted, reliance being placed wholly on the distinctiveness of the typhoid colony. Such attempts, however, in many comparative tests, have shown very poor results. Many of the lactose-fermenting types simulate the colony of the typhoid bacillus so closely that great difficulty is encountered; this is not the case with plates inoculated with the same material, but with the indicator and the lactose present. As has been noted, if colon types develop partially on the dye agar, they will simulate the typhoid colonies. Similarly, many types which develop with a high dilution of the dye, but fail to develop at lower dilutions, show a typhoid-like colony. It would seem then, that the lactose aids in promoting the maximal development of those lactose-fermenting types which are able to develop at all. When this is the case, they do not resemble the typhoid colonies, altho they may when their growth is restrained. This deduction seems warranted, as the greatest trouble is noted with the weaker dye agar, when lactose is omitted, whereas the troublesome types are excluded to a great extent on the stronger dye agar. As has been noted, the slight color developing in the typhoid colonies adds to their distinctiveness, but should it be absent, typhoid-like colonies are, as a rule, not confusing unless the lactose be omitted.

Some of the slow lactose-fermenting types which, under certain circumstances, develop typhoid-like colonies, change the indicator very slightly. This change, however, is sufficient to rob them of most of their typhoid-like appearance.

The reason for the 2 concentrations of the dye has already been fully discussed. The results show that both dilutions are necessary, as the stronger may give so great a degree of inhibition that the typhoid bacilli are lost.

One brand of brilliant green has been used in most of this work. Considerable variation is noted at times with certain solutions of the dye or certain batches of agar. A cloudy agar with sediment cannot be used, as the dye loses a large part of its activity. Different samples of the same brand of dye probably vary and the one brand of dye may not always be available. For this reason it was necessary to determine some method of standardization that could be used not only to test a sample of dye, but also to check the concentration of a dye solution with each batch of agar. When many routine examinations are being made, this takes comparatively little time, and the necessity of repeating the tests can be avoided by preparing the agar in large amounts.

Various methods for standardization were employed, using carrier stools, various freshly isolated strains, as well as stock cultures of typhoid bacilli. As the amount of extraneous matter in the stools was a disturbing factor and the various strains showed some variations in resistance, the Rawlings strain, which is available in most laboratories, was finally adopted for the comparative tests. The amount of culture inoculated is important, as overseeding will obscure the gradations in the activity of the dye. Table 5 shows the results of a few standardization tests with varying amounts inoculated, these results serving as examples of the action of a correctly balanced dye agar.

TABLE 5
Results of Standardization Tests

Endo	Amount of Dye to 100 c.c. Agar		
	0.2 c.c. Dye	0.3 c.c. Dye	0.5 c.c. Dye
Thousands	Thousands	Thousands	75% reduction
Thousands	Thousands	Slight reduction	90% reduction
2,000	2,000	1,500	No growth
200	200	180	No growth
60	60	50	No growth
40	40	35	No growth
30	25	6	No growth

In making the tests, the plates are streaked, but with a little practice fairly uniform inoculations can be obtained. In obtaining dilutions for inoculation, we have found that 0.5 c.c. of a broth culture diluted with 10 c.c. of broth will contain 2,000 to 5,000 bacilli per small loop. If a small loop of a broth culture be added to 10-15 c.c. of broth, a loop will contain from 25 to 200 bacilli.

If the dilutions given are tried, it is a simple matter to test out a sample of dye or a batch of agar. Should variations be noted from the results given, proportionate changes in the final dilutions of the dye to be employed can easily be determined. The fact that 2 dilutions are used compensates for slight variations.

For an occasional examination with an untested sample of dye the use of 0.2, 0.3, and 0.4 c.c. of the dye to 100 c.c. of agar would probably cover the range of action of most samples of brilliant green or variations in media.

Three samples of dye, from Bayer, Grübler, and Höchst, have been compared by this method, 2 solutions being used of each dye made from different

parts of the sample. The variations between the different dyes were moderate and no greater than between the two solutions made from the same dye. With careful standardization it would seem, therefore, that any of these preparations could be used. If the test against the Rawlings strain gives correct results as far as we can see, the restraining action upon the fecal flora will be satisfactory. The degree of restraint of the fecal flora varies with different stools. The degree of restraint is, as a rule, greater with fresh specimens. In becoming familiar with the medium, we strongly advise the use of stools artificially inoculated with traces of carrier stools or with typhoid culture. The use of pure cultures may give misleading results, as fecal strains belonging to the B. coli group vary widely.

The dye solutions keep fairly well, but there seems to be some irregularity in their rate of deterioration. We have used some solutions as long as 2 months. It would be well, however, to re-test a solution every 2 weeks as a safeguard against deterioration. The poured plates keep for several days. We have not tested this point as it is relatively unimportant to those doing routine examinations.

ROUTINE METHOD NOW EMPLOYED

A large sample of the stool to be examined is rubbed up in extract broth and diluted with broth to a density (roughly a 1 : 15 dilution by volume of a formed stool) which, experience has shown, will give discrete colonies when a loopful is streaked over 2 successive Endo plates. The suspension is allowed to stand so that particles of feces will settle. One loop of the suspension is placed on a 0.2-c.c. and on a 0.3-c.c. plate; these are streaked in the order given and then an Endo plate. Two loops are placed on each of a similar pair of green-dye plates and streaked in the same order, and last an Endo plate. The wire used for streaking is not flamed while inoculating the series of plates. For practical routine examinations this seems a sufficient number of plates to employ. Altho the Endo plates have never been positive when the dye agar was negative, we do not feel with our present experience that they can be omitted. Unusually sensitive strains are a possibility.

Some of the details of our technic might be described, as we find that they are not generally known. We do not employ glass rods to inoculate the plates, as they are unwieldy and cool too slowly after sterilization, but a suitably bent, heavy platinum wire with a loop at the end. A loop of the material is carried across the center of the plate and then smeared over the plate with the flat part of the wire, the plate being turned so that the entire surface is sown. A second plate can be inoculated in the same fashion without burning the wire. If 3 such wires are employed and used in rotation, they cool before being required again.

For a direct tentative agglutination from the plate, the macroscopic slide method is employed. A small drop of salt solution and one of an agglutinating serum are placed on a slide. The suspicious colony is taken up with a very small loop and rubbed first in the salt solution and then in the serum. A positive result is shown by immediate clumping, whereas the salt control remains evenly clouded. If spontaneous agglutination occurs, this will be noted in the control. A high titer serum is essential and the dilution must be lower than that ordinarily employed, as the reaction must take place very rapidly or the drop will dry. The optimal dilution must, of course, be determined for each sample of serum. As the fecal strains of typhoid are more agglutinable than blood strains, inagglutinable strains are not so apt to be encountered. One peculiar source of error has been noted, however. Certain strains have shown a prezone phenomenon; that is, they agglutinated poorly or not at all at low dilutions, but agglutinated well at higher dilutions. However, this method, with a known serum, gives results that are very reliable and we give a tentative diagnosis on this basis. Unknown sera, especially if they be fresh, may lead to serious error unless thoroughly tested with various types developing on the plates, as well as with known strains of allied bacilli.

For fishing, the Russell medium is employed with the following modification. In place of litmus, 1% of the Andrade indicator is used, and the reaction set accordingly. This modified medium gives much sharper results, and the acid production in the butt is very evident; the indicator does not decolorize, as is the case with litmus. As the medium is rather delicate, each new lot should be tested, before use, with a few known strains, to be sure that it is not too sensitive.

In fishing from the green-dye agar there is the possibility of not obtaining pure cultures. This is a disadvantage of all differential restraining media, the restrained bacteria being able to grow when transferred to a favorable medium. It is well to save suspicious plates until the fishings are inspected, or to replate from them immediately.

Besides inoculating plates in the manner described, 0.1 c.c. of the fecal suspension may be added to 1% glucose extract broth, neutral to phenolphthalein, containing 1 : 300,000 of the dye. If, as occasionally happens, very little growth develops on the direct dye agar and no typhoid is found, Endo agar is inoculated from the broth tube after 18 hours' incubation. This is most likely to be successful when the flora is dye-susceptible, giving an added chance of success. Should

the dye agar, however, show abundant growth, sub-inoculations from the dye-broth tube seem superfluous.

There would seem to be no reason why these results cannot be applied to the isolation of members of the paratyphoid-enteritidis groups. We have had only one naturally infected specimen,—a sample of soup which produced gastro-enteritis in several members of a family. When this was plated on Endo, about 1,000 colonies developed, of which 1 was a paratyphoid type. On green-dye plates of the usual strength, only a few paratyphoid types developed.

Pure cultures of this group were plated with seeding well under 150 for a plate, to determine whether appreciable restraint would be evident on the green-dye agar of 0.2, 0.3, and 0.5 c.c. strength.

Most of the strains showed no, or at most slight, inhibition on the strongest dye agar. Some showed a moderately marked inhibition, and a few failed to grow on the 0.3 c.c. dye agar. A few showed inhibition even on the 0.2 c.c. dye agar. This agrees with our previous conclusion[2] that the members of this group have a variable sensitiveness to brilliant green, altho most of the strains are extremely dye-resistant.

The colony morphology is variable. The more feebly growing types are typhoid-like; the more vigorous strains produce 2 kinds of colonies with intermediate gradations. Both types are larger than the typhoid colony. One type of colony is yellowish-white, raised, and moist. The denser center is homogeneous, but the edge shows some markings similar to those in the typhoid colony. The other type is flattened, spreading, feathery, and irregular in outline, the typhoid-like markings being exaggerated. Some strains produced both types of colonies, and fishings on replating bred true.

The use of dye broth for enrichment is more likely to be successful with the resistant members of this group, as stronger dilutions of the dye may be employed.[2]

We have not discussed the results of previous investigations of the application of aniline dyes for the enrichment of the typhoid-paratyphoid group. Our own work developed independently on the basis of our observations of the action of the various aniline dyes. As the outcome has shown, it is fortunate that we did not attempt merely to modify existing media. We have tested several of the methods suggested, with results inferior to our own. No attempt was made to try out the various malachite-green media, as our results have shown that this dye is inferior to brilliant green in differential action. Furthermore, different samples of malachite green have been found to vary widely in their action, whereas most samples of brilliant green give similar results.

As the Conradi brilliant-green agar[4] approached most nearly the medium we have devised, we have employed it in comparative tests. The results, given in the preceding tables, are not very favorable to Conradi medium. Furthermore, this latter, containing 3% of agar (which seems to us unnecessary), and 20 grams of meat extract to the liter (whereas we used only 3), is more difficult to make and more expensive—both factors of importance in routine work. On the whole the Conradi medium is too restraining; the colonies are somewhat characteristic but insufficiently so. German workers agree with us in these points. The Conradi agar is now chiefly employed as a preliminary enrichment medium. The plates are heavily inoculated and after incubation are flooded with broth, whereupon the typhoid colonies, less adherent than other types, float off easily, and the suspension is inoculated either on Conradi-Drigalsky medium or on Endo. We have tried this method with different green-dye media, and altho the results were better than from the use of Endo medium and the usual technic, they were not as good as with our medium alone. Furthermore, this method delays the results one day.

China green has been employed by Werbitski, and some workers have found it superior to the Conradi brilliant-green medium as a preliminary enrichment medium. We did not find China green more differential in its action than brilliant green; moreover, its action is not evident at equally high dilutions. For this reason, we have not tried this medium and are inclined to attribute any better results obtained in its use as referable to a better adjustment of the dilution of the dye to the other constituents rather than to any inherent superiority in the dye itself.

The attempts of various workers[4] to use a differentially selective dye decolorized by sodium sulfite and designed to act both as a restraining agent and indicator, are, in our opinion, due to a misconception of the mode of action of the dyes employed. We have found it impossible to balance the degree of loss of action of the dye due to sodium sulfite. We have also employed caffein, as advised by some investigators, with only moderate success.

It would require too much space to analyze even a small part of the literature which exists on typhoid isolation. The mere existence of a great number of methods is evidence of the unsatisfactory results obtained. On the whole, however, the application of the selective media described in this paper has been very successful in the isolation of the more resistant paratyphoid-enteritidis group.

SUMMARY

With an easily prepared agar, 2 concentrations of brilliant green being used, it has been possible markedly to increase the percent of positive typhoid isolations from stools. This has given much better results than the employment of brilliant-green broth alone, altho the dye-broth enrichment method may give positive results when the agar alone fails. The methods can be applied to the isolation of the members of the paratyphoid enteritidis group from stools.

[4] Kutscher: Kolle and Wassermann, Handb. d. pathogen. Mikroorganismen, 1913, 3, p. 717.

THE MECHANISM OF THE ABDERHALDEN REACTION WITH BACTERIAL SUBSTRATES *

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Since the appearance of Abderhalden's dialysis reaction and his explanation of the mechanism as a digestion of substrate by serum ferments, various phenomena have been observed which throw some doubt on the validity of this theory. The fact that a positive reaction, as evidenced by an increase in dialyzable, ninhydrin-reacting substances, may result from the combination of inorganic materials with serum, indicates that his explanation does not cover all conditions. It is obvious that the positive reaction in this case cannot be the result of the digestion of substrate. It may be assumed rather that the reaction is primarily an adsorption process, the secondary stage of which is the autodigestion of the serum protein by ferments inherent in the serum itself. In fact, proof of this hypothesis has been offered by many observers, among whom are De Waele,[1] Plaut,[2] Bronfenbrenner[3] and Jobling, Eggstein, and Petersen.[4]

While arriving at the same conclusion in regard to the fact that the Abderhalden reaction is essentially dependent upon an adsorption process, these investigators hold different views in regard to individual factors influencing the reaction. De Waele[1] considers that the mechanism of the reaction consists in a change in the state of solubility of the serum globulins. This change may be due to the physical action of non-specific inorganic substances, or it may be produced in a specific manner by the interaction of a serum and its homologous substrate. He places emphasis upon the fact that the reaction, under certain conditions, may be specific. Specificity, however, should be ascribed, not to a multiplicity of individual ferments, but to factors which modify the state of solubility of the globulins.

Plaut[2] also suggests that organic substrates may induce through physical processes the formation of substances which will react positively to ninhydrin.

Jobling, Eggstein, and Peterson[4] are convinced that no actual digestion of substrate can take place. In support of this view they have demonstrated that placental tissue, when subjected to treatment with pregnant serum, presents a definite increase in nitrogen-containing material. This increase is due to an adsorption of antitrypsin, as is indicated by the fact that placenta which has remained in contact with pregnant serum, is subsequently more resistant to tryptic digestion than normal placenta. Thus any digestion which may occur

[1] Ztschr. f. Immunitätsf. O., 1914, 22, p. 170.
[2] München. med. Wchnschr., 1914, 61, p. 238.
[3] Jour. Exper. Med., 1915, 21, p. 221.
[4] Ibid., p. 239.

is due, not to the activity of specific ferments upon their substrates, but to the serum proteases, which, following the adsorption of the inhibitory agents (antiferments), are free to act upon the serum proteins.

Bronfenbrenner[3] agrees with the view of the last-mentioned investigators that the reaction is a phenomenon of adsorption. With inorganic substrates it depends simply upon physical adsorption of serum antiferments, as by kaolin, barium sulfate, or substances of a similar nature. With organized substrates a more complicated mechanism is involved. This, Bronfenbrenner has resolved into 2 distinct phases. The first comprises a sensitization of the substrate by the specific elements of the immune serum with the resultant adsorption of antitrypsin. The second is the autodigestion of the serum.

Employing placental tissue and pregnant serum, Bronfenbrenner found that the reaction was specific, but that the specificity was limited entirely to the first phase of the reaction. This he demonstrated by allowing a serum and substrate to remain in contact in the cold, no digestion occurring. When, however, serum and substrate were separated and the serum was allowed to dialyze alone at incubator temperature, a positive reaction was obtained in those cases in which the serum was specific. That a change in the serum had occurred was evident. That a change had also taken place in the substrate was shown through the fact that thenceforth this sensitized substrate induced the appearance of dialyzable products in any serum. In the specificity of substrate sensitization he sees a phenomenon analogous to an antigen-antibody reaction — an observation which had already been made by De Waele.[1]

As Bronfenbrenner's work had been confined entirely to tissue substrates, it was considered of interest to determine whether the same principles were operative in the case of bacterial substrates, and whether the limits of specificity were as clearly defined.

Accordingly, the experiments here to be reported were conducted in strict conformity with the technic of Bronfenbrenner. Immune sera were obtained from rabbits which had received repeated injections of typhoid bacilli ("Rawling's"), paratyphoid A bacilli, and Staphylococcus aureus. Duplicate animals were immunized with each strain. The bacterial substrates were prepared as in work previously reported by Smith.[5] The dialysis thimbles were Schleicher and Schüll's No. 579A, and had been tested for both permeability and impermeability.

The rabbits were bled from 5 to 7 days after the last injection and the serum of each rabbit was combined with its homologous substrate and also with the two non-specific substrates. For each serum there was a control tube with no substrate. Controls were also made upon substrates alone. The serum-substrate contacts, as well as the controls, were held in the cold for 16 hours. The tubes were then centrifugated and during centrifugation they were kept packed in ice that there might be no possibility of a digestion of the serum during the interval required for its complete separation from the substrate. After centrifugation the clear serum was removed from the substrate and held in the ice-box until it was transferred to dialyzing sacs. Dialysis was allowed to proceed at 37 C. for 16 hours, after which the usual ninhydrin test was made. The substrates which had been collected by centrifugation were washed twice in normal salt solution to remove all traces of serum. Each of the substrates was then divided into 4 portions, to 3 of which fresh serum was added

⁵ Jour. Infect. Dis., 1915, 16, p. 319.

from the three types of immunized rabbits, and to the fourth portion fresh serum of a normal rabbit. These were again allowed to remain in contact in the cold for 16 hours. The tubes were then centrifugated cold; the serum was removed, placed in dialyzing sacs, and left at incubator temperature for 16 hours. The dialysates were tested for the presence of substances reacting positively to ninhydrin.

The serum of each of the immune animals, as well as that of 2 normal rabbits, was subjected to this treatment. The procedure applied to Rabbit 16, immunized to typhoid, represents a typical test. For clearness the procedure is presented in the following tables and is divided into the two phases.

TABLE 1

Procedure I—First Phase: Serum and Substrate Mixed and Placed on Ice for 16 Hours; then the Mixtures were Centrifugated in the Cold, and the Serum Dialyzed for 16 Hours at 37 C.

| Tube | July 7 | | July 9 | |
	Serum (1 c.c. + Substrate (2 c.c.)		Ninhydrin Test	Sac
a............................	16	Typhoid....................	+	a
a............................	16	Typhoid....................	+	a
b............................	16	Paratyphoid A............	—	b
b............................	16	Paratyphoid A............	—	b
c............................	16	Staphylococcus aureus....	—	c
c............................	16	Staphylococcus aureus....	—	c
d (control)...............	16	None........................	—	d
e (control).............	None	Typhoid....................	—	e
f (control).............	None	Paratyphoid A............	—	f
g (control).............	None	Staphylococcus aureus....	—	g

TABLE 2

Procedure II—Second Phase: The Two Tubes of Each Type of Substrate were United, Washed Twice with Salt Solution, Divided into 4 Portions, and Combined with Fresh Serum from Rabbits 16 (Typhoid), 18 (Paratyphoid A), 20 (Staphylococcus Aureus), and 22 (Normal). These Mixtures were then Placed on Ice 16 Hours; then Centrifugated in the Cold, and the Serum Dialyzed 16 Hours at 37 C.

| Tube | July 8 | | July 10 | |
	Serum (1 c.c. + Substrate (1 c.c.)		Ninhydrin Test	Sac
1........................	16	Typhoid a*................	+	1
2........................	16	Paratyphoid A b..........	—	2
3........................	16	Staphylococcus aureus c..	—	3
4........................	16	None........................	—	4
5........................	18	Typhoid a.................	+	5
6........................	18	Paratyphoid A b..........	+	6
7........................	18	Staphylococcus aureus c..	—	7
8........................	18	None........................	—	8
9........................	20	Typhoid a.................	+	9
10.......................	20	Paratyphoid A b..........	—	10
11.......................	20	Staphylococcus aureus c..	+	11
12.......................	20	None........................	—	12
13.......................	22	Typhoid a.................	+	13
14.......................	22	Paratyphoid A b..........	—	14
15.......................	22	Staphylococcus aureus c..	—	15
16.......................	22	None........................	—	16

* The letter following the name of the substrate indicates the tube in Procedure I from which the substrate was taken. For example, Typhoid "a" represents a typhoid substrate which has already been in contact with a typhoid serum.

It is evident from the work outlined in Procedure I that an immune serum, when combined in the cold with its specific substrate and subsequently dialyzed alone, undergoes some degradation, as is indicated by the positively reacting dialysates in Sacs "a." This same serum, when combined in an identical manner with heterologous substrates, as in Tubes "b" and "c," is not digested, nor is there any digestion in Control Tubes "d" and "e." Obviously, during the contact in the cold the specific substrate enters into some reaction with the serum. That this reaction is dependent upon specific factors is indicated by the fact that no change occurs in the serum after contact with non-specific substrates.

Evidence that the interaction between specific substrate and serum may be of the nature of a specific adsorption follows from a consideration of Procedure II. The substrate designated as Typhoid "a," which has been in contact with a typhoid serum in a specific union, now so acts upon all the sera—typhoid, paratyphoid A, Staphylococcus aureus, and normal—that each serum upon subsequent dialysis gives a positive reaction (Sacs 1, 5, 9, and 13). From this we must conclude that Substrate Typhoid "a," through contact with its specific serum in Procedure I, has acquired a property which it did not inherently possess; namely, the property of causing a positive ninhydrin reaction with any serum. That this change is to be referred only to the specific action of the serum on the substrate is proved by the fact that no similar change occurs in paratyphoid A or Staphylococcus aureus substrates. In fact, these substrates, which have been treated with a non-specific serum (typhoid), have remained unchanged in the property of failing to produce a positive reaction in any serum except their homologous sera (Paratyphoid A, 2, 6, 10, 14; and Staphylococcus aureus 3, 7, 11, 15).

Note should be made of the fact that the previous contact of a substrate with a non-specific serum in no way impairs its activity when subsequently combined with a specific serum (Sacs 6 and 11).

Table 3 summarizes the results obtained with the entire series. It will be noted in Table 3 that each serum presents results analogous to those given in detail in Tables 1 and 2. It is evident that the sera of animals which have received the same immunizing treatment—15 and 16 with typhoid, 17 and 18 with paratyphoid A, 19 and 20 with Staphylococcus aureus—and also the two normal sera react in an

identical manner. Whatever the initial combination of serum and substrate, as long as the union is specific, a sensitization of substrate occurs. Whenever the primary contact occurs between a substrate and heterologous or normal sera, such a sensitization invariably fails to become manifest.

TABLE 3

Summary of Results in Procedures I and II

Procedure I		Ninhydrin Test	Procedure II								
				Serum							
Serum	Substrate*		Substrate†	15	17	19	21	16	18	20	22
15	Typhoid.........	+	Typhoid a........	+	+	+	+				
15	Paratyphoid A..	−	Paratyphoid A b	−	+	−	−				
15	S. aureus........	−	S. aureus c.......	−	−	+	−				
15	None............	−	None.............	−	−	−	−				
16	Typhoid.........	+	Typhoid a........	..	..	..	..	+	+	+	+
16	Paratyphoid A..	−	Paratyphoid A b	..	..	..	..	−	+	−	−
16	S. aureus........	−	S. aureus c.......	..	..	..	..	−	−	+	−
16	None............	−	None.............	..	..	..	..	−	−	−	−
17	Typhoid.........	−	Typhoid a........	+	−	−	+‡				
17	Paratyphoid A..	+	Paratyphoid A b	+	+	+	+				
17	S. aureus........	−	S. aureus c.......	−	−	+	−				
17	None............	−	None.............	−	−	−	−				
18	Typhoid.........	−	Typhoid a........	..	..	..	..	+	−	−	−
18	Paratyphoid A..	+	Paratyphoid A b	..	..	..	..	+	+	+	+
18	S. aureus........	−	S. aureus c.......	..	..	..	..	−	−	+	−
18	None............	−	None.............	..	..	..	..	−	−	−	−
19	Typhoid.........	−	Typhoid a........	+	−	−	−				
19	Paratyphoid A..	−	Paratyphoid A b	−	+	−	−				
19	S. aureus........	+	S. aureus c.......	+	+	+	+				
19	None............	−	None.............	−	−	−	−				
20	Typhoid.........	−	Typhoid a........	..	..	..	..	+	−	−	−
20	Paratyphoid A..	−	Paratyphoid A b	..	..	..	..	−	+	−	−
20	S. aureus........	+	S. aureus c.......	..	..	..	..	+	+	+	+
20	None............	−	None.............	..	..	..	..	−	−	−	−
21	Typhoid.........	−	Typhoid a........	+	−	−	−				
21	Paratyphoid A..	−	Paratyphoid A b	−	+	−	−				
21	S. aureus........	−	S. aureus c.......	−	−	+	−				
21	None............	−	None.............	−	−	−	−				
22	Typhoid.........	−	Typhoid a........	..	..	..	..	+	−	−	−
22	Paratyphoid A..	−	Paratyphoid A b	..	..	..	..	−	+	−	−
22	S. aureus........	−	S. aureus c.......	..	..	..	..	−	−	+	−
22	None............	−	None.............	..	..	..	..	−	−	−	−

* Sera and substrates in contact on ice 16 hr. Substrates separated by centrifugation and washed twice with salt solution. Sera dialyzed 16 hr.
† Substrates from Procedure I placed in contact with fresh sera on ice 16 hr. Centrifugated cold. Sera dialyzed 16 hr.
‡ An aberrant positive reaction for which no explanation can be given.

SUMMARY

The present work, confirming the studies of Bronfenbrenner, demonstrates that the Abderhalden reaction may be divided into 2 phases. The first phase involves a sensitization of substrate by its

specific serum. The second phase represents an autodigestion of the serum, which is not due to specific causes.

In the sensitization of substrate an absolute specificity obtains. Whether or not this sensitization is of the nature of an antigen-antibody reaction forms at present the subject of a further investigation.

STUDIES IN NON-SPECIFIC COMPLEMENT FIXATION *

I. NON-SPECIFIC COMPLEMENT FIXATION BY NORMAL RABBIT SERUM

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The object of this communication is to direct attention to the observation that in complement-fixation tests when normal rabbit serum is used, according to the usual technic, in an active condition or after heating at 55 C. for half an hour for the purpose of inactivation, non-specific complement absorption or fixation may occur, not only in the presence of lipoidal antigens, but with various bacterial antigens as well. This is a matter of considerable importance in view of the fact that rabbits are commonly employed for experimental studies in syphilis, and likewise for the preparation of immune amboceptors for the study and differentiation of bacterial species and proteins in general. This possibility of non-specific complement fixation is to be remembered and reckoned with in complement-fixation work with rabbit serum, especially in experimental syphilis, as otherwise erroneous conclusions may be drawn, if based alone upon complement-fixation tests.

HISTORICAL REVIEW

Schilling and Hoesslin[1] were probably among the first investigators to observe that normal rabbit serum may absorb or fix complement with alcoholic extracts of syphilitic liver; Manteufel and Woithe[2] and Browning and McKenzie[3] have also noted the phenomenon during studies in experimental trypanosomiasis. Dohi[4] examined the sera of 74 normal rabbits, using as antigen an alcoholic extract of syphilitic liver, and found that 39 reacted positively and 35 negatively. He also observed that heated serum was more likely to show this non-specific absorption of complement than unheated serum. Browning and McKenzie, however, state that they have not observed this phenomenon when using serum in a fresh, or active, condition. Blumenthal[5] has likewise observed positive reactions with normal rabbit serum, using an alcoholic extract of syphilitic liver as antigen; Craig and Nichols[6] on the other hand have reported

[1] Deutsch. med. Wchnschr., 1908, 34, p. 1422.
[2] Centralbl. f. Bakteriol., R., 1909, 43. p. 359.
[3] Jour. Path. and Bacteriol., 1909-1911, 15, p. 182.
[4] Beitr. z. path. u. Therap. der Syph., 1911, p. 514.
[5] Berl. klin. Wchnschr., 1911, 48, p. 1462.
[6] Jour. Exper. Med., 1911, 14, p. 206.

uniformly negative results with an alcoholic extract of syphilitic liver. Similar observations on this power of normal rabbit serum to absorb complement in the presence of lipoidal antigens have been made by Emmanuel[7] and by Epstein and Pribram,[8] the former stating that the administration of salvarsan removes this property temporarily and the latter making similar claims for the mercurials.

Kolmer with Casselman[9] studied this subject, testing the sera of 117 normal and apparently healthy rabbits with antigens comprising alcoholic extracts of syphilitic liver, extracts of acetone insoluble lipoids, alcoholic extract of beef heart re-enforced with cholesterin, and aqueous and alcoholic extracts of a pure culture of Treponema pallidum. Inactivated serum was used throughout. After heating the sera at 55 C. for half an hour, doses varying from 0.05 to 0.4 c.c. being employed, the following results were obtained:

(a) In doses of 0.05 c.c. serum, complement fixation did not occur with any of the antigens employed.

(b) In doses of 0.1 c.c. serum, complement fixation occurred in 13% of sera with alcoholic extracts of syphilitic liver and in 20% with extracts of beef heart re-enforced with cholesterin.

(c) In doses of 0.2 c.c., fixation occurred in 20% of sera with alcoholic extracts of syphilitic liver; in 33% with extracts of acetone insoluble lipoids; and in 40% with cholesterinized alcoholic extracts of heart. With larger doses of serum, as 0.4 c.c., the percentage of complement fixations with the various antigens was even higher.

We have examined the sera of a number of apparently normal rabbits in both the fresh or active, and heated or inactivated state, not only with various lipoidal antigens commonly used in the Wassermann syphilis reaction, but with several indifferent bacterial antigens as well, and we believe that these results are of such importance and significance as to merit a brief report.

METHODS OF STUDY

Sera.—The sera of 75 unused and apparently normal rabbits were used. These animals were kept on a diet composed largely of cabbage, oats, and hay; blood was secured from time to time from an ear vein, and, as a general rule, all sera were tested within 24 to 48 hours, both in an active and inactivated state.

Antigens.—The following three lipoidal extracts were used: alcoholic extracts of human and beef heart re-enforced with cholesterin; an alcoholic extract of syphilitic liver; an extract of acetone insoluble lipoids from human heart. These lipoidal extracts were the same as those used in the regular Wassermann syphilis reactions and were used in the same dosage; namely, twice the antigenic unit as determined by titration with the sera of several luetic persons. These doses were at least 6 to 12 times less than the anticomplementary units as determined by frequent titrations.

For the preparation of bacterial antigens we used the following three micro-organisms: 3 strains of Staphylococcus pyogenes aureus; 1 strain of

[7] Berl. klin. Wchnschr., 1911, 48, p. 2335.

[8] Ztschr., f. exper. Path. u. Therap., 1909, 7, p. 549.

[9] Jour. Med. Research, 1913, 28, p. 369.

TABLE 1

Number and Percentage of Positive Reactions With Active and Inactivated Rabbit Sera and Various Lipoidal and Bacterial Antigens

Antigens	Active Serum			Inactivated Serum		
	No. Positive	No. Negative	Percentage Positive	No. Positive	No. Negative	Percentage Positive
Cholesterinized alcoholic extract of heart........	11	62	15	37	38	49.3
Alcoholic extract of syphilitic liver	6	67	8.2	30	43	41
Acetone insoluble lipoids .	4	69	5.4	28	46	38.3
Staphylococcus	15	20	42.8	22	13	62.8
B. coli	11	24	31.4	18	17	61.4
B. typhosus	13	22	37.1	21	14	60

B. typhosus; 6 strains of B. coli communis from human feces. These micro-organisms were not selected for any other reason than that they are easily cultivated and represent common species.

These bacterial antigens were simple suspensions of the washed bacteria, shaken for 24 hours in salt solution, filtered through paper, heated to 60 C. for an hour, and preserved with 0.5% phenol. All of them were titrated each time before the tests proper for their anticomplementary units and used in one-quarter amounts.

TABLE 2

Degree of Complement Fixation with the Various Lipoidal and Bacterial Antigens with Active and Inactivated Normal Rabbit Sera

Antigens	Active Sera				
	No. Positive	Less than 25% Inhibition	25% Inhibition	50% Inhibition	75% Inhibition
Cholesterinized alcoholic extract of heart..	11	1	2	5	1
Alcoholic extract of syphilitic liver........	6	0	2	1	2
Acetone insoluble lipoids.................	4	0	2	1	0
Staphylococcus	15	1	2	5	5
B. coli	11	3	4	2	0
B. typhosus	13	3	3	3	4

The dosage of either a lipoidal or bacterial antigen bears an important relation to the occurrence and degree of complement absorption, for larger doses of antigen, as one-half the anticomplementary unit, will yield a higher percentage of positive reactions than a dose representing one-quarter or less of this unit.

Technic.—As a general rule, the sera were used in a constant dose of 0.1 to 0.2 c.c.; when inactivated a temperature of 55-56 C. was employed with an exposure of one-half hour. With 0.2 c.c. serum, the percentage of positive reactions and degree of complement absorption were considerably higher than those occurring with 0.1 c.c.

For complement, the pooled sera of 2 or more guinea-pigs were used, diluted 1:20 with normal salt solution, and employed in a constant dose of 1 c.c. The sheep hemolytic system was used throughout, the hemolytic amboceptor being titrated each day with this dose of complement and 1 c.c. of a 2.5% suspension of washed cells, and used in the main tests in a dose equal to 2 hemolytic units. In this manner the hemolytic system is adjusted for each complement serum and erythrocyte suspension. As will be pointed out later, the technic is a matter of importance, as a greater excess of complement or hemolysin yields a lower percentage of positive reactions, whereas with the single unit system the percentage of positive reactions becomes much higher.

Serum complement and antigen with sufficient normal salt solution were incubated for an hour at 37 C.; 2 units of hemolysin and 1 c.c. of the corpuscle suspension were then added. After a second incubation of from 1 to 2 hours, the readings were made. As usual a serum control was used in every instance; likewise antigen, hemolytic, complement, and corpuscle controls as is our custom in conducting complement-fixation tests with human serum. In all tests the sera were used in a constant dose of 0.1 c.c.

SUMMARY OF RESULTS

As shown in the summary of these tests (Table 1) complement absorption under the technic described, with the use of fresh, active, normal rabbit serum and lipoidal extracts, was found to occur in from 5 to 15% of sera; when these same sera were inactivated by heating

TABLE 2—*Continued*

Degree of Complement Fixation with the Various Lipoidal and Bacterial Antigens with Active and Inactivated Normal Rabbit Sera

100% Inhibition	50% or Less Inhibition	Inactivated Sera						
		No. Positive	Less than 25% Inhibition	25% Inhibition	50% Inhibition	75% Inhibition	100% Inhibition	50% or Less Inhibition
3	72	37	2	5	12	6	13	51.3
1	50	30	0	5	15	3	7	66.6
1	75	28	5	5	10	2	6	71.4
2	53.3	22	0	1	5	5	11	27.3
2	81.8	18	0	9	6	1	2	82.3
0	84.6	21	0	1	7	2	11	38.1

them in a water bath at 55-56 C. for 30 minutes, complement absorption was observed in 38-49% of sera.

Of equal, or even more, significance is the observation that with various bacterial antigens fresh, active, normal rabbit serum showed some degree of complement absorption in 31-42% of sera; inactivated sera in 51-62%.

As shown in Table 2, the degree of complement absorption with lipoidal extracts was usually moderate or slight, in that 50-75% of

sera showed 50%, or less, inhibition of hemolysis. Extracts re-enforced with cholesterin are most likely to react strongly when they react at all.

Bacterial antigens showed even slighter degrees of complement absorption, in that 50-85% gave 50%, or less, inhibition of hemolysis.

CONSTANCY OF RABBIT SERUM IN RELATION TO NON-SPECIFIC COMPLEMENT FIXATION

In order to determine whether the reaction with a rabbit serum was permanent or subject to change under natural conditions, 15 rabbits were examined at intervals of 3 days to a month. Of these, 12 rabbits showed persistently negative or persistently positive results. Three showed varying results as shown in Table 3. As must be

TABLE 3

VARIATION IN REACTION OF THE SAME RABBIT SERUM AT DIFFERENT TIMES

Serum	Active Serum (0.1 c.c.)				Inactivated Serum (0.1 c.c.)			
	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids	Serum Control
19	—	—	—	—	—	—	—	—
19	—	—	—	—	++	++	++	—
19	—	—	—	—	—	—	—	—
19	—	++	—	—	—	++	—	—
6	++	—	—	—	++++	++++	++++	—
6	—	—	—	—	++++	++++	++++	—
23	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—
23	0	0	0	0	++	++++	+	—
23	0	0	0	0	++++	++++	+++	—
35	—	—	—	—	++++	++	++	—
35	—	—	—	—	+++	+++	+++	—
35	—	—	—	—	±	±	—	—
35	0	0	0	0	++++	++++	++++	—
35	0	0	0	0	++++	++++	++++	—
35	—	—	—	—	++	+++	++	—

expected among the positively reacting animals, very slight differences in the degrees of inhibition of hemolysis were noted at different times, as, for example, a ++ reaction becoming a + or a +++ a week later and subsequently ++ again, or even ++++, but a positively reacting rabbit tends to remain so, as is likewise true with a negatively reacting rabbit.

DISCUSSION

An adequate explanation of this phenomenon of non-specific complement fixation with normal rabbit serum has not been made. As

already stated, coccidiosis bears no relation to the condition. It is to be remembered that dosage of serum, kind and dosage of antigen, the degree of temperature and length of exposure in inactivation, and especially the adjustment of the hemolytic system, are factors modifying the occurrence and degree of this non-specific complement absorption. These and other factors that have been subjected to study will be discussed in a separate paper; here it may be stated that while it is well known that the serum alone or an antigen alone may under certain circumstances show non-specific complement absorption, being the so-called "anticomplementary" action of serum or antigen, we are certain that in the technic employed these factors have been adequately controlled and that the results cannot be ascribed to anticomplementary action of serum or to the employment of a too closely adjusted hemolytic system. Indeed, all these examinations were made bi-weekly along with a large number of Wassermann reactions with human sera in which the same lipoidal antigens and same hemolytic systems were employed. The phenomenon is of practical interest and importance, not only from the standpoint of complement-fixation tests in experimental studies in rabbits, but also because a study and explanation of its mechanism may throw more light upon the nature of the syphilis reaction.

We believe that this property of rabbit serum of absorbing or fixing complement in a non-specific manner should be emphasized and better known, for when this animal is used for the purposes of immunization with the object of subsequently conducting complement-fixation tests with the serum, the factor of non-specific complement fixation enters and may greatly modify the interpretation of results. This is of importance not only in experimental studies, but in medico-legal work, as in the differentiation and identification of proteins by complement-fixation tests. It is our custom to test rabbits one or more times before immunization, preferably with the antigens to be subsequently used in the complement-fixation tests, and select those that show a persistently negative reaction.

SUMMARY

Fresh, active sera from normal rabbits in doses of 0.1 c.c. showed non-specific complement fixation with lipoidal extracts in 5-15% of sera.

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When these same sera were inactivated by heating them in a water bath at 55-56 C. for 30 minutes, complement fixation occurred in 38-49% of sera.

With both active and inactivated sera the highest percentage of positive reactions was observed when alcoholic extracts of heart muscle re-enforced with cholesterin were used as antigens; alcoholic extracts of syphilitic liver gave the second highest, and an extract of acetone insoluble lipoids gave the lowest, percentage of positive reactions.

With bacterial antigens of staphylococci and colon and typhoid bacilli, fresh, active rabbit sera showed some degree of complement fixation in 31-42% of instances; when these sera were inactivated, positive reactions occurred in 51-62%.

The degree of complement fixation with normal rabbit serum and lipoidal and bacterial antigens is usually moderate or slight, as about 50-85% of reactions show 50%, or less, inhibition of hemolysis.

The reacting state of a rabbit's serum when the animal is on an average diet is usually constant, in that 80% of our animals reacted persistently negatively or persistently positively when examined at intervals of 3 days to a month.

It is to be emphasized that when rabbits are employed for experimental studies with a view to using their sera for complement-fixation tests, their sera should be tested one or more times, before inoculation, preferably with the particular antigen to be used, and only those selected that react negatively.

STUDIES IN NON-SPECIFIC COMPLEMENT FIXATION*

II. NON-SPECIFIC COMPLEMENT FIXATION BY NORMAL DOG SERUM

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The present investigation was rendered necessary by our researches in distemper and intestinal parasitism of dogs, both of which included complement-fixation tests, in order to ascertain whether apparently normal and healthy dog serum will fix or absorb complement with lipoidal and bacterial antigens as will normal rabbit serum.[1]

Several years ago Rossi[2] observed that a large percentage of the sera of normal dogs yield non-specific complement-fixation with watery extracts of syphilitic liver as antigen, but this observation has not attracted much attention, probably on account of the relative infrequency with which complement-fixation tests are conducted with dog sera.

During the past year we have tested the sera of about 200 dogs with both lipoidal and various bacterial antigens, with the result that a large percentage were found capable of bringing about some degree of non-specific absorption of complement in a manner comparable to that of normal rabbit serum. Tho perfectly fresh and active normal dog serum may give non-specific complement fixation, the tendency is much increased by heating sera at 56 C. for 30 minutes. During the process of heating (inactivation) normal dog serum quickly develops antihemolytic or anticomplementary properties and indeed this condition may occasionally be found in perfectly fresh serum; but the object of this communication is to direct attention to the observation that the sera of normal dogs may show positive or non-specific complement-fixation when this antihemolytic or anticomplementary property is not in evidence.

METHOD OF STUDY

The technic employed was exactly similar to that used by us in conducting the Wassermann syphilis reaction with human serum and

[1] Kolmer and Casselman: Jour. Med. Research, 1913, 28, p. 369. Kolmer and Trist: Jour. Infect. Dis., 1916, 18, p. 20.

[2] Ztschr. f. Immunitätsf., R., 1909, 1, p. 429.

in a similar study of non-specific complement fixation with normal rabbit serum.

Sera.—The animals were kept on an ordinary mixed diet and bled from the external jugular vein. The sera were tested while in a fresh, active condition, and again after heating at 56 C. for half an hour. In the majority of tests the sera were used in 0.1 c.c. amounts, but for purposes of a quantitative study a number were tested in amounts of 0.05, 0.1, 0.2, and 0.4 c.c.

Many of the dogs examined showed some evidences of distemper, and it is not improbable that a proportion of the older animals had distemper prior to admission to the kennels, but in complement-fixation the sera of young and apparently healthy dogs showed in a similar manner a tendency to absorb complement with various lipoidal and bacterial antigens, so that in this study we have included all animals under the heading of "normal," altho distemper or some other infection prior to these tests may have had an influence upon the results with our bacterial antigens. This subject will be discussed in a later communication; here are recorded the results of complement-fixation tests with the sera of dogs of various ages ordinarily met with in the kennels.

Antigens.—Both lipoidal extracts and bacterial emulsions were employed. The lipoidal extracts were the three used in our routine Wassermann reactions: an alcoholic extract of human heart re-enforced with cholesterin; an alcoholic extract of syphilitic liver; and an extract of acetone insoluble lipoids. The doses of these, determined at intervals by titration with luetic sera, equalled twice their antigenic units. These doses were at least from 6 to 10 times less than the anticomplementary units.

Three bacterial antigens were employed; namely, polyvalent emulsions of washed 24-hour agar cultures of staphylococci, human colon bacilli, and typhoid bacilli. The staphylococcus antigen was prepared from 5 strains of Staphylococcus aureus from abscesses and 6 strains of Staphylococcus albus from acne vulgaris—all from human infections; the colon antigen was prepared from 6 strains of B. coli communis from human feces and the typhoid antigen from 2 strains of B. typhosus. The emulsions were shaken mechanically for several hours, filtered through paper, heated at 60 C. for an hour, and preserved with 0.25% phenol. Each of these antigens was titrated before the complement-fixation tests, and used in amounts equalling one-quarter of their anticomplementary doses.

The doses of antigen employed are important as the percentage of positive reactions may be altered by using amounts larger or smaller than those mentioned.

Technic.—The antisheep hemolytic system was used throughout. Complement was furnished by the pooled sera of 2 or more guinea-pigs and used in dose of 1 c.c. of a 1:20 dilution (0.05 c.c. undiluted serum). The hemolysin was titrated in increasing doses against this unit of complement serum and 1 c.c. of a 2.5% suspension of washed sheep erythrocytes, prepared for each day's work and used in the antigen titrations and main tests in an amount equal to double the hemolytic unit. Less than this amount of hemolysin increases the percentage of positive reactions and the anticomplementary tendencies of the serum alone.

Serum, antigen, complement, and sufficient salt solution were incubated for an hour at 37 C.; then 2 units of hemolysin and 1 c.c. of the erythrocyte suspension were added; after mixing, the tubes were re-incubated for an hour or an hour and a half, depending upon the hemolysis of the controls, and then

placed in the refrigerator over night, the readings being made the following morning. As customary, a control on every serum, antigen, and complement, and hemolytic controls, were included.

SUMMARY OF RESULTS

Dog serum in both an active or fresh condition and after heating or inactivation is capable of absorbing complement, in a relatively large percentage of instances, with various lipoidal and bacterial antigens.

The animals tested were those ordinarily met with in the kennels, and while a number of these had or probably had had distemper, most of them, tested within a short time after admission, showed no evidences of this infection. In this series no differences in complement fixation with the antigens employed were noted between the sera of normal dogs and the sera of those showing the symptoms of distemper, and the results have been ascribed to a process of non-specific complement fixation or absorption.

TABLE 1

The Percentages of Positive Reactions with Increasing Doses of Active Dog Serum and Various Lipoidal and Bacterial Antigens

Antigen	Percentage of Positive Reactions			
	0.05 c.c. Serum	0.1 c.c. Serum	0.2 c.c. Serum	0.4 c.c. Serum
Cholesterinized alcoholic extract of human heart............	29	16	0	0
Alcoholic extract of syphilitic liver	25	8	0	0
Acetone insoluble lipoids.......	12.5	4	0	0
Staphylococci	96	54	75	25
B. coli (human)...............	90	66	50	0
B. typhosus	91	70	41	0

As shown in Tables 1 and 2, the highest number of positive reactions was observed with the bacterial antigens. With heated sera from normal dogs it was the exception rather than the rule to observe a negative reaction with these antigens and this has a very important bearing upon the question of complement-fixation tests with dog serum for diagnostic purposes, as in the complement-fixation test for distemper.

Among the lipoidal extracts, the highest percentage of positive reactions occurred with alcoholic extract of human heart re-enforced with cholesterin; next in the order of yielding positive reactions came an alcoholic extract of syphilitic liver, while an extract of

acetone insoluble lipoids yielded the lowest percentage of positive reactions. These relations held with both active and inactivated sera, and the results are similar to those observed with normal rabbit serum. It would appear that the presence of cholesterin in an extract increases the percentage of positive reactions, as it undoubtedly enhances the antigenic sensitiveness of any tissue extract for the syphilis reagin in the Wassermann reaction.

As shown in Table 1, fresh, active dog serum in dose of 0.05 c.c. yielded the highest percentage of positive reactions with both lipoidal and bacterial antigens; while with doses of 0.2 and 0.4 c.c. of serum no reactions occurred with the lipoidal extracts, and with the bacterial antigens the tendency for complement fixation was greatly reduced. It is probable that native hemolytic complement and antisheep hemolysin present in the dog sera were partly responsible for these results and obscured lesser degrees of complement absorption.

TABLE 2

THE PERCENTAGES OF POSITIVE REACTIONS WITH INCREASING DOSES OF HEATED (INACTIVATED) DOG SERUM AND VARIOUS LIPOIDAL AND BACTERIAL ANTIGENS

Antigen	Percentages of Positive Reactions			
	0.05 c.c. Serum	0.1 c.c. Serum	0.2 c.c. Serum	0.4 c.c. Serum
Cholesterinized alcoholic extract of human heart..............	39	63	60	50
Alcoholic extract of syphilitic liver	25	50	48	50
Acetone insoluble lipoids.......	26	21	36	30
Staphylococci	90	96	100	70
B. coli (human)...............	73	80	88	50
B. typhosus	40	76	87	70

Heating dog serum at 55 C. for 30 minutes greatly increases the tendency toward non-specific complement fixation, not only with lipoidal, but also with the bacterial, antigens. This is at once apparent in comparing Tables 1 and 2. The tendency toward absorption or fixation of complement and the percentage of positive reactions increase with increasing doses of serum, except when 0.4 c.c. serum is used, when the tendency somewhat diminishes as a result to some degree of a masking of complement fixation by the presence of natural or normal antisheep hemolysins in dog serum.

The degree or amount of complement absorption with lipoidal extracts is usually moderate or slight, as measured by the method

employed. This is shown in Table 3, which summarizes the percentages of positive reactions showing 50%, or less, inhibition of hemolysis with the various antigens. With the cholesterinized alcoholic extract of heart and alcoholic extract of syphilitic liver, more complement was absorbed than with the extract of acetone insoluble lipoids; the bacterial antigens showed a greater degree of complement absorption inasmuch as the reactions were more frequently $+++++$ and $+++$ than $++$ or less.

TABLE 3

THE PERCENTAGES OF POSITIVE REACTIONS OF 50% ($++$), OR LESS, INHIBITION OF HEMOLYSIS WITH 0.1 C.C. OF ACTIVE AND INACTIVATED DOG SERA AND VARIOUS ANTIGENS

Antigens	Serum (0.1 c.c.)	
	Active	Inactivated
Cholesterinized alcoholic extract of human heart..............	70	43
Alcoholic extract of syphilitic liver.........................	77	68
Acetone insoluble lipoids....................................	100	94
Staphylococci	45	32
B. coli (human)...	41	42
B. typhosus	28	50

DISCUSSION

Without discussing at this time the mechanism of non-specific complement fixation with dog serum, we would point out the tendency which dog serum shows in this connection, and urge great caution in the conduct and interpretation of complement-fixation tests with it.

While dog serum is not infrequently antihemolytic or anticomplementary after inactivation, even when fresh and shortly after bleeding, we have not included any of these sera in this work, so that the positive reactions are to be interpreted as evidences of complement fixation or absorption between the antigens and some substance in the serum. As with rabbit serum, dog serum develops this property of fixing complement in a non-specific manner as the result of heating the serum at 56 C. for one-half hour, altho fresh, active serum may also show the phenomenon, as indicated in the tables.

When it is desired to conduct complement-fixation tests with dog serum for specific amboceptors, it would appear advisable to use the serum in a perfectly fresh and active condition in dose of 0.1 to 0.2 c.c.; or, after heating the serum at 62 C. instead of 55 C. for half an hour, since this, as will be pointed out later, removes, or greatly diminishes, the tendency toward non-specific fixation of complement.

STUDIES IN NON-SPECIFIC COMPLEMENT FIXATION *

III. THE INFLUENCE OF SPLENECTOMY AND ANESTHETICS ON THE NON-SPECIFIC COMPLEMENT FIXATION SOMETIMES SHOWN BY NORMAL RABBIT AND DOG SERA

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This investigation was undertaken in the hope of obtaining some understanding of the part played by the spleen in hemolysis and of the mechanism by which the red cells develop an increased resistance to hemolytic agents after splenectomy. Antischkow[1] has shown that the feeding of rabbits with cholesterin leads to an increased deposition of anisotropic fats in the spleen. Eppinger[2] and King[3] have found that dog's blood after splenectomy shows an increase in total fats and cholesterin and a decrease in unsaturated fatty acids.

These observations naturally suggest that if the spleen has such an important influence on fat and lipoid metabolism, this mechanism may in some way be concerned in the changes which the spleen effects in the red cells. That the change in the red cells which is responsible for their increased resistance to hemolytic agents after splenectomy is a characteristic of the cells themselves and not a change in the serum, is indicated by the work of Karsner and Pearce,[4] tho it must be admitted in view of King's results that it may be an antihemolytic power of the serum, dependent upon an increase in cholesterin content.

As stated in the first paper[5] of this series, several investigators have noted the occurrence in presumably normal rabbits of positive complement fixation with lipoidal antigens. The reason for this is not known, but we have evidence at hand that lipoidal substances

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* Received for publication August 8, 1915.

[1] Ueber experimentell erzeugte Ablagerungen von Anisotropen Lipoidsubstanz in der Milx und im Knockenmark, Beitr. z. path. Anat. u. allg. Path., 1913, 88, p. 201.

[2] Zur Pathologie der Milzfunktion, Berl. klin. Wchnschr., 1913, 50, pp. 1509, 1572.

[3] Studies in the Pathology of the Spleen, Arch. Int. Med., 1914, 14, p. 145.

[4] The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. IV. A Study by the Methods of Immunology of the Increased Resistance of the Red Cells after Splenectomy, Jour. Exper. Med., 1912, 16, p. 769.

[5] Jour. Infect. Dis., 1916, 18, p. 20.

in the serum are concerned in this process, and in view of the well-known importance of lipoids in the Wassermann reaction, it occurred to us, in connection with Anitschkow's observations, as well as those of Eppinger and King, that if in the sera of rabbits showing this phenomenon the reaction became negative after splenectomy, it might be possible by chemical analysis of erythrocytes and serum, and by influencing the reaction through feeding experiments, to throw some light upon the relation of the spleen to normal hemolysis and also upon the question of the factors responsible for increased resistance of the erythrocytes after splenectomy. Our results have not supported our hypothesis; for, altho splenectomy does cause weakening and sometimes a temporary disappearance of the property in normal dog and rabbit serum of non-specific complement fixation, the important factor in this appears to be the anesthetic and not the absence of the spleen. Our observations, however, are of some interest in connection with the problems concerning the mechanism of the Wassermann reaction, and are for that reason presented in brief at this time.

In the present communication are presented only observations of the influence of splenectomy and various anesthetics on the process of non-specific complement fixation in normal rabbit and dog sera. Other antilytic studies and feeding experiments will be published in the fourth paper of this series.

Technic and Method of Study

The rabbits were kept on a constant diet of cabbage, oats, and hay; the dogs were given the usual mixed diet of meats, bread, and vegetables.

By means of preliminary complement-fixation tests, animals the sera of which yielded strongly positive reactions with the antigens used were selected for these studies. Following these selections, the usual procedure was to secure a specimen of blood immediately before anesthesia and operation, and again at varying intervals after operation. The sera were tested in the same dosage, with the same antigens, and with the same technic as in the pre-operative tests; in this manner the influence of the anesthetic and splenectomy upon the serum reactions was studied.

The complement-fixation tests were conducted with sera in a fresh, active state, and again after heating or inactivation at 56 C. for half an hour. The sera were generally used in dosage of 0.1 c.c.; a few experiments were conducted with 0.2 c.c. The dosage of serum appears to us to be important, especially after inactivation, as with the larger dose the percentage of positive reactions is higher and the influence of various factors less marked.

All sera were examined with the 3 lipoidal extracts used in our regular routine Wassermann reactions: an alcoholic extract of beef heart re-enforced with cholesterin; an alcoholic extract of syphilitic liver; and an extract

of acetone insoluble lipoids of human heart. All these extracts were used in dosage equal to twice their antigenic units as determined by titration, these doses being from 6 to 12 times less than their anticomplementary units. In this manner false positive reactions due to antilytic properties of the antigens were avoided. In addition to these lipoidal extracts, 2 bacterial antigens of staphylococci and Bacillus coli communis from human sources were used in our later experiments because of the previous observations that a large percentage of normal rabbit and dog sera absorbed complement with various indifferent and non-specific bacterial antigens. These antigens were titrated prior to each set of reactions and used in the main tests in doses representing one quarter of their anticomplementary units.

The antisheep hemolytic system was employed. Complement was furnished by the pooled sera of at least 2 guinea-pigs and used in dosage of 1 c.c. of a 1 : 20 dilution ($=$ 0.05 c.c. undiluted serum). The hemolysin was titrated in ascending doses against this constant dose of complement and corpuscle suspension (1 c.c. of a 2.5%) and 2 hemolytic units in the tests proper.

TABLE 1

INFLUENCE OF ETHER ANESTHESIA AND SPLENECTOMY ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

| Rabbit | Date | Reaction Before Operation | | | | | | | |
| | | Active Serum | | | | Inactivated Serum | | | |
		Choles-terinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Choles-terinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
1	Feb. 6	—	—	—	—	++++	++	++	—
3	Feb. 6	—	—	—	—	+	++	±	—
6	Feb. 19	—	—	—	—	++++	++++	++++	—
	Mar. 4	—	—	—	—	++++	++++	++++	—
7	Feb. 19	—	—	—	—	++++	++++	++++	—
	Mar. 4	—	—	—	—	++++	++++	++++	—
9	Feb. 19	—	—	—	—	++++	++++	++++	—
15	Feb. 6	—	—	—	—	++++	—	—	—
42†	April 6	—	—	—	—	++++	++++	++++	—
	April 8	—	—	—	—	+++	++	++	......

† Serum from Rabbit 42 was tested in dosage of 0.2 c.c. with all antigens.

KEY TO TABLES

++++ $=$ complete inhibition of hemolysis (strongly positive).
+++ $=$ 75% inhibition of hemolysis (moderately positive).
++ $=$ 50% inhibition of hemolysis (weakly positive).
+ $=$ 25% inhibition of hemolysis (very weakly positive).
± $=$ less than 25% inhibition of hemolysis.
D. H. $=$ delayed hemolysis.
— $=$ complete hemolysis.

As usual antigen, hemolytic, and corpuscle controls were included and invariably a serum control on each serum. The latter is especially important in conducting complement-fixation tests with dog sera because of their tendency to present antihemolytic properties.

All tests were made bi-weekly at the same time as our regular Wassermann reactions, which carefully controlled the antigens and hemolytic system.

INFLUENCE OF ETHER ANESTHESIA AND SPLENECTOMY ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

The results are shown in Table 1. As a general rule, the operation of splenectomy required from 12 to 20 minutes, during which the animal was under full ether anesthesia.

TABLE 1—*Continued*

Influence of Ether Anesthesia and Splenectomy on Non-specific Complement Fixation with Normal Rabbit Serum

Date of Opera-tion	Date	Reaction After Operations							
		Active Serum				Inactivated Serum			
		Choles-terinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Choles-terinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
Feb. 16	Feb. 23	—	—	—	—	—	—	—	—
	Mar. 4	—	—	—	—	++++	+++	++	—
	Mar. 9	—	—	—	—	++++	+++	+++	—
Feb. 16	Feb. 23	—	—	—	—	—	—	—	—
	Mar. 4	—	—	—	—	+	+++	+	—
	Mar. 9	—	—	—	—	+	+++	+	—
Mar. 7	Mar. 9	—	—	—	—	++++	++++	++	—
	Mar. 16	—	—	—	—	++++	+++	+++	+++
	Mar. 23	+	+	+	—	—	+	+	—
Mar. 7	Mar. 9	—	—	—	—	—	—	—	—
	Mar. 16	—	—	—	—	—	—	—	—
	Mar. 23	—	—	—	—	—	—	—	—
Feb. 26	Mar. 4	—	—	—	—	++++	++++	++++	—
	Mar. 9	—	—	—	—	++++	+++	±	—
	Mar. 16	—	—	—	—	++	++	+	—
	Mar. 23	—	—	—	—	++	+++	+	—
Feb. 16	Feb. 23	—	—	—	—	—	—	—	—
	Mar. 4	—	—	—	—	+++	—	—	—
—	April 8	—	—	—	—	++	++	++	—
	April 10	—	—	—	—	++	++	++	—
	April 13	—	—	—	—	++	++	++	—

As shown in the table, 7 rabbits were examined before, and at intervals after, operation. Of these the sera of 4 (Rabbits 1, 3, 7, and 15) did not fix complement during the following week or 10 days, while 2 (6 and 42) showed a somewhat weaker reaction, and one (9) no effect at all.

INFLUENCE OF ETHER ANESTHESIA AND SPLENECTOMY AND ETHER
ANESTHESIA ALONE ON NON-SPECIFIC COMPLEMENT FIXA-
TION WITH NORMAL DOG SERUM

Similar results were observed in dogs, as shown in Table 2. Three
dogs were tested before splenectomy and ether anesthesia; in the sera
of all three there was complement fixation to some extent with one or
more antigens. After splenectomy serum tests were conducted at

TABLE 2

INFLUENCE OF ETHER ANESTHESIA AND SPLENECTOMY ON NON-SPECIFIC COMPLEMENT FIXATION WITH
DOG SERUM

Dogs	Date	Reaction Before Operation							
		Active Serum				Inactivated Serum			
		Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
14–17	Feb. 10	+	±	—	—	+ +	+	±	±
14–18	Feb. 10	+ +	+	—	—	+ + + +	+ + + +	+ + + +	+ + +
14–19	Feb. 10	+ + +	+ + +	+ + +	—	+ + + +	+ + + +	+ + + +	+ + +
14– 3	....	....	....	....	....	....	....	....	......
13–88	....	....	....	....	....	....	....	....	......
13–84	....	....	....	....	....	....	....	....	......
13–83	....	....	....	....	....	....	....	....	......
13–87	....	....	....	....	....	....	....	....	......
13–85	....	....	....	....	....	....	....	....	......
13– 9	....	....	....	....	....	....	....	....	......
12–51	....	....	....	....	....	....	....	....	......

varying intervals up to about a month after operation; in all tests
complement fixation remained persistently absent or was much weaker.
In this respect a more profound influence was noted in dogs after
splenectomy under ether anesthesia than in rabbits, and, while the
results may not be attributable entirely to the anesthetic, we are inclined
to ascribe a large part of the serum changes to this agency, as will
be pointed out later in this paper.

In addition, 8 dogs were examined one or more times after splenectomy under ether anesthesia, performed from 1 to 10 months previously. As these animals were splenectomized before this work was begun, serum tests were not made preliminary to the operations, but the sera of these animals yielded in general fewer positive reactions than those observed among a large series of normal dogs (6), altho under the circumstances the changes are not sufficiently pronounced

TABLE 2—*Continued*

INFLUENCE OF ETHER ANESTHESIA AND SPLENECTOMY ON NON-SPECIFIC COMPLEMENT FIXATION WITH DOG SERUM

Date of Operation	Date	Reaction After Operations							
		Active Serum				Inactivated Serum			
		Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
Feb. 19	Feb. 23	—	—	—	—	—	—	—	—
	Mar. 12	—	—	—	—	+	—	—	—
	Mar. 19	—	—	—	—	—	—	—	—
	Mar. 23	—	—	—	—	—	—	—	—
Feb. 19	Feb. 23	—	—	—	—	±	—	—	—
Feb. 19	Feb. 23	—	—	—	—	=	—	—	—
	Mar. 12	—	—	—	—	—	—	—	—
	Mar. 19	—	D. H.	—	—	—	—	—	—
	Mar. 23	D. H.	—	—	—	D. H.	—	—	—
Feb. 19	Mar. 19	—	—	—	—	—	—	—	—
	April 2	++++	++++	++++	±	++++	++++	++++	+
Feb. 12	Mar. 16	—	—	—	—	—	—	—	—
Jan. 7	Mar. 10	—	—	—	—	—	—	—	—
	April 2	—	—	—	—	—	—	—	—
Dec. 10	Mar. 19	—	—	—	—	—	—	—	—
	April 2	++++	++++	++++	—	++++	++++	++++	±
Dec. 12	Feb. 10	—	—	—	—	+	+	+	±
	Mar. 18	—	—	—	—	—	—	—	—
Dec. 10	Feb. 10	++++	+	—	—	++++	++++	++++	+++
	Mar. 19	—	—	—	—	—	—	—	—
	April 2	±	—	—	—	+	±	—	—
April 9	Feb. 10, 1914	—	—	—	—	—	—	—	—
	Mar. 19	—	—	—	—	—	—	—	—
May 31, '14	Feb. 10, 1914	—	—	—	—	+	+	+	+
	Mar. 19	—	—	—	—	—	—	—	—
	Mar. 26	—	—	—	—	D. H.	—	—	—

to lay particular stress on the possible influence of the removal of the spleen.

As controls on the relation of the absence of the spleen to these results, we have studied the influence of (1) ether anesthesia alone and of ether anesthesia and nephrectomy on the serum reactions of a number of rabbits; (2) splenectomy under chloroform anesthesia and chloroform alone; (3) splenectomy under urethan anesthesia and

TABLE 3

Influence of Ether Anesthesia Alone on Non-specific Complement Fixation with Normal Rabbit Serum

		Reaction Before Ether Anesthesia											
		Active Serum						Inactivated Serum					
Rabbit	Date	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control
25	Dec. 10	++	++	—	0	0	—	++++	++++	++++	0	0	—
26	Dec. 10	+++	+++	++	0	0	—	++++	++++	++++	0	0	—
29	Dec. 10	++	++	—	0	0	—	+++	+++	++	0	0	.....
30	Dec. 12	—	—	—	0	0	—	—	—	—	0	0	—
35	Dec. 12	—	--	---	0	0	—	++++	++++	+	0	0	—
38	Dec. 12	—	—	—	0	0	—	--	—	—	0	0	—
1*	Mar. 9	—	--	—	0	0	—	++++	++++	+++	0	0	—
15*	Mar. 4	—	—	--	0	0	—	+++	—	—	0	0	—
18	Mar. 26	+++	+++	+++	0	0	—	+++	+++	+++	0	0	—
34†	Mar. 30	++++	+++	0	++++	++++	—	++++	++++	0	++++	++++	—
	April 1	++++	+++	0	++++	++++	—	++++	++++	0	++++	++++	—
36	Mar. 30	—	—	0	++++	++++	—	++++	+++	0	++++	++++	—
	April 1	—	—	0	++	+++	—	++++	+++	0	++++	++++	—
44	April 8	+++	+++	0	++++	++++	—	++++	++++	0	++++	++++	—
51	April 23	—	—	0	—	—	—	+++	+++	0	+++	+++	—
	May 3	--	—	0	—	—	—	+++	+++	0	+++	+++	—
52	April 23	—	—	0	—	—	—	++++	++++	0	++++	++++	—
	May 3	—	—	0	—	—	—	++++	++++	0	++++	++++	—
54	April 23	—	—	0	—	—	—	++++	++++	0	++++	++++	--
	May 3	—	—	0	—	—	—	++++	++++	0	++++	++++	—

* Rabbits 1 and 15 had been splenectomized (see Table 1).
† The sera of Rabbits 34, 36, 44, 51, 52, and 54 were used in dosage of 0.2 c.c. in all tests.

TABLE 3—*Continued*

INFLUENCE OF ETHER ANESTHESIA ALONE ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

| | | Reaction After Ether Anesthesia | | | | | | | | | | | |
| | | Active Serum | | | | | | Inactivated Serum | | | | | |
Duration of Anesthesia	Date	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control
½ hr.	Dec. 10	++	++	—	0	0	—	+++	+++	++	0	0	—
	Dec. 14	—	—	—	0	0	—	+	+	—	0	0	—
	Dec. 17	+	±	—	0	0	—	++	++	++	0	0	—
½ hr.	Dec. 10	—	—	—	0	0	—	++	++	+	0	0	—
½ hr.	Dec. 10	++	++	—	0	0	—	+++	++	+	0	0	—
	Dec. 14	—	—	—	0	0	—	+	+	—	0	0	—
	Dec. 17	++	—	—	0	0	—	+++	+++	++	0	0	—
½ hr.	Dec. 12	—	—	—	0	0	—	—	—	—	0	0	—
	Dec. 14	—	—	—	0	0	—	—	—	—	0	0	—
	Dec 17	—	—	—	0	0	—	—	—	—	0	0	—
½ hr.	Dec. 12	—	—	—	0	0	—	++++	++++	+	0	0	—
	Dec. 14	—	—	—	0	0	—	+++	+++	—	0	0	—
	Dec. 17	—	—	—	0	0	—	++++	++++	++++	0	0	—
½ hr.	Dec. 12	—	—	—	0	0	—	—	—	—	0	0	—
	Dec. 14	—	—	—	0	0	—	—	—	—	0	0	—
Mar. 17 1½ hr. Mar. 17	Mar. 23	—	—	—	0	0	—	--	±	—	0	0	—
Mar. 17 ½ hr. Mar. 30	Mar. 23	—	—	—	0	0	—	+	—	—	0	0	—
	April 23	—	—	—	0	0	—	—	—	—	0	0	—
½ hr.	April 2	+++	+++	+++	0	0	—	+++	+++	++	0	0	+
	April 6	++	++	++	0	0	—	+++	+++	+++	0	0	—
½ hr.	April 1	++++	++++	0	++++	++++	—	++++	++++	0	++++	++++	—
	April 2	—	—	0	—	+	—	—	—	0	++	++	—
	April 3	++++	++++	0	++++	++++	—	++++	++++	0	++++	++++	—
	April 6	++++	++++	0	++++	++++	—	++++	++++	0	++++	++++	—
10 min.‡	April 1	—	—	0	—	—	—	++++	++++	0	++++	++++	—
25 min.	April 13	—	—	0	—	—	—	++++	++++	0	++	++	—
	April 16	—	—	0	—	—	—	++++	+++	0	+++	+++	—
May 3 § 10 min.	May 3	—	—	0	—	—	—	—	—	0	—	—	—
May 3 10 min.	May 3	—	—	0	—	—	—	++++	++++	0	++++	++++	—
	May 4	—	—	0	—	—	—	++++	++++	0	++++	++++	—
	May 5	—	—	0	—	—	—	++++	++++	0	++++	++++	—
	May 6	—	—	0	—	—	—	++++	++++	0	++++	++++	—.
May 3 10 min.	May 3	—	—	0	—	—	—	++++	++++	0	++++	++++	—
	May 4	—	—	0	—	—	—	++++	++++	0	+++	++++	—
	May 5	—	—	0	—	—	—	++++	++++	0	++++	++++	—
	May 6	—	—	0	—	—	—	++++	++++	0	++++	++++	—

‡ Died during a 10-minute period of anesthesia. Blood was at once secured.
§ Died 4 minutes after anesthesia. Blood was at once secured.

urethan alone; (4) splenectomy under nitrous oxid anesthesia and nitrous oxid alone.

INFLUENCE OF ETHER ANESTHESIA ALONE ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

Fifteen rabbits in all were examined before, and at intervals after, ether anesthesia varying from 10 to 30 minutes in duration. The results are shown in Table 3.

1. The sera of 5 rabits (25, 29, 1, 15, and 34) showed a definite weakening or absence of complement fixation, with both active and inactivated sera, at some time during a brief period following ether anesthesia.

2. The sera of 3 rabbits (26, 36, and 44) showed changes when tested in an active state but not after heating or inactivation.

TABLE 4

INFLUENCE OF ETHER ANESTHESIA AND SPLENECTOMY ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

| Rabbit | Date | Reaction Before Operation | | | | | | | |
| | | Active Serum | | | | Inactivated Serum | | | |
		Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
18	Mar. 26	+++	+++	+++	—	+++	+++	+++	—
21	Mar. 30 April 2	++++ ++++	++++ ++++	++++ ++++	— —	++++ ++++	++++ ++++	++++ ++++	+++ ++

3. The sera of 4 rabbits (35, 18, 52 and 54) did not appear to be affected at all.

4. The influence of ether alone is only slight immediately after the anesthesia, and is usually most marked after several days. Gradually the serum returns to its former condition in relation to non-specific complement fixation.

5. As already pointed out, changes in the power of rabbit serum to give these non-specific complement fixations due to ether anesthesia are more apparent when it is used in a fresh, active state; likewise, in a number of reactions it would appear that of the lipoidal extracts the reaction with an alcoholic extract of heart re-enforced with cholesterin showed least change, and the altered power of

reaction of a serum was less in evidence with the bacterial antigens than with the lipoidal antigens.

It may be noted in this connection that the effect of ether anesthesia upon rabbit serum in the Wassermann reaction is directly opposite to that described as occurring with human serum, inasmuch as a positively reacting rabbit serum tends to become negative. The mechanism of non-specific complement fixation with rabbit and dog sera, however, presents several features differing widely from that concerned in the Wassermann reactions with human serum, so that, while no doubt somewhat related, they are not identical.

It would appear, therefore, that the results obtained after splenectomy are due to anesthesia rather than to removal of the spleen. This supposition was strengthened when nephrectomy under ether anesthesia in 2 rabbits showed similar changes (Table 4).

TABLE 4—*Continued*

Influence of Ether Anesthesia and Nephrectomy upon Non-specific Complement Fixation with Normal Rabbit Serum

Date of Operation	Date	Reaction After Operation							
		Active Serum				Inactivated Serum			
		Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
April 11	April 23	—	—	—	—	—	—	—	—
	May 5	—	—	—	—	—	—	—	—
	May 11	—	—	—	—	—	—	—	—
	Oct. 16	—	—	—	—	—	—	—	—
April 7	April 13	—	—	—	—	+++	++	+	—
	April 23	+++	++	++	—	+++	++	++	—

INFLUENCE OF CHLOROFORM ANESTHESIA AND SPLENECTOMY AND CHLOROFORM ANESTHESIA ALONE ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

Changes similar to those following ether anesthesia were apparent after the administration of chloroform. Our series, however, does not include a large number of animals because of the difficulty in giving chloroform to rabbits over a prolonged period of time.

The results in serum changes following one splenectomy under chloroform anesthesia and those following chloroform anesthesia alone are shown in Table 5. Unfortunately the splenectomized animal succumbed soon after operation, so that a series of examinations could not

be made. While in this rabbit changes in the reacting power of the serum occurred—positive reactions with the lipoidal antigens becoming negative, and those with the bacterial antigens weaker after the anesthesia and operation—the influence of chloroform alone was somewhat less apparent than had been the influence of ether alone, probably, to some extent, on account of the shorter period of anesthesia.

TABLE 5

Influence of Chloroform Anesthesia and Splenectomy and Chloroform Anesthesia Alone on Non-specific Complement Fixation with Normal Rabbit Serum

| | | Reaction Before Anesthesia and Operation | | | | | | | | | | | |
| | | Active Serum | | | | | | Inactivated Serum | | | | | |
Rabbit	Date	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control
43*	April 6	0	0	0	0	0	0	++++	++++	0	++++	++++	—
	April 8	—	—	0	—	—	—	++++	++++	0	++++	++++	—
39	Mar. 30	—	—	0	++++	++	—	++	++	0	++++	++	.....
	April 1	—	—	0	—	—	—	+++	+++	0	++++	+++	—
40	Mar. 30	—	—	0	++++	++	—	+	++	0	++++	++	—
	April 1	—	—	0	++++	++	—	+	++	0	++++	++++	—
45	April 8	--	—	0	—	—	—	++	++	0	++++	++++	—

* All tests with the sera of these rabbits were made with 0.2 c.c. amounts.

INFLUENCE OF URETHAN ANESTHESIA AND SPLENECTOMY AND URETHAN
ALONE ON NON-SPECIFIC COMPLEMENT FIXATION WITH
NORMAL RABBIT SERUM

Urethan was administered in dose of 15 grains to each rabbit by means of a stomach tube. The effects upon the serum reactions were similar to those observed following the administration of ether, probably, in part, on account of a similar solvent action of the drug upon lipoids.

The results of the complement-fixation tests are shown in Table 6. In the two splenectomized animals (Rabbits 24 and 30), the

serum reactions were altered soon after operation, becoming weaker or negative and later running to their former states. Likewise, after the administration of urethan alone (Rabbits 32 and 34), the reactions were much weaker and in this respect were similar to the changes produced by ether, in that the alteration of serum reaction was not immediate, but was apparent one or two days after an interval of 7 to 10 days.

TABLE 5—*Continued*

INFLUENCE OF CHLOROFORM ANESTHESIA AND SPLENECTOMY AND CHLOROFORM ANESTHESIA ALONE ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

Anesthesia and Duration of Operation	Date	Reaction After Anesthesia and Operation											
		Active Serum						Inactivated Serum					
		Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Staphylococci	Colon Bacilli	Serum Control
Splenectomy†	April 8	—	—	0	—	—	—	—	—	0	++	++++	—
Chloroform 6 min.	April 1	--	—	0	—	—	—	—	--	0	—	—	—
Chloroform 10 min.	April 1	—	...	0	+	+	—	+	+++	0	++++	++++	--
	April 2	—	---	0	+	++	—	0	0	0	0	0	0
	April 3	—	--	0	—	—	—	++	++	0	++++	++++	—
	April 6	—	—	0	—	—	—	+	+	0	+	+	—
	April 10	—	—	0	—	—	—	++	++	0	++++	++++	—
	April 13	—	—	0	—	—	—	++	++	0	++++	++++	—
	April 16	—	—	0	—	—	—	++	++	0	++	++	—
Chloroform 10 min.	April 8	—	—	0	—	—	—	±	±	0	++++	++++	—
	April 13	—	—	0	—	—	—	++	++	0	++++	++++	—
	April 16	—	—	0	—	—	—	+++	++	0	++++	++++	—

† Duration of anesthesia 15 minutes.

INFLUENCE OF NITROUS OXID ANESTHESIA AND SPLENECTOMY AND NITROUS OXID ANESTHESIA ALONE ON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM

In order to settle more definitely the question of the influence of removal of the spleen on the phenomenon of non-specific complement fixation in normal rabbit serum, 2 positively reacting animals were splenectomized under nitrous oxid oxygen anesthesia; in this manner we avoided using anesthetics that are lipoid solvents, as ether, chloroform, and urethan. These operations required about 12 minutes each,

during which the animals were completely anesthetized. For controls, 2 other positively reacting rabbits were anesthetized in the same manner and for the same length of time.

Serum tests were made immediately before and after the anesthesia and operations, and again at intervals of several days. No appreciable changes in the reaction followed splenectomy under nitrous oxid oxygen anesthesia. Likewise, the anesthetic alone had no influence upon the

TABLE 6

Influence of Urethan Anesthesia and Splenectomy and Urethan Anesthesia Alone on Non-specific Complement Fixation with Normal Rabbit Serum

Rabbit	Date	Reaction Before Anesthesia and Operation							
		Active Serum				Inactivated Serum			
		Choles-terinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Choles-terinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
24	Nov. 3	—	—	—	—	++++	++	++	—
30	Nov. 3	—	—	—	—	++++	++++	++++	—
32	Nov. 3	—	—	—	—	+++	+++	++	—
34	Nov. 3	—	—	—	—	++++	++	++	—

serum reactions. The conclusion seems warranted, therefore, that the results observed after splenectomy under ether, chloroform, and urethan are to be ascribed to the anesthetic used rather than to the removal of the spleen itself and are due probably to the effects of the former upon serum lipoids.

CONCLUSIONS

Anesthetics as ether, chloroform, and to a slight extent urethan, generally weaken or remove temporarily the power in normal rabbit

and dog sera of absorbing or fixing complement with lipoidal and bacterial antigens in a non-specific manner. This alteration usually is not apparent at once after the administration of the anesthetic, but is found after 1 to 3 days; later the serum returns to its former power of causing this non-specific complement fixation.

The administration of ether does not alter negatively reacting sera in such manner as to bring about positive reactions.

TABLE 6—*Continued*

Influence of Urethan Anesthesia and Splenectomy and Urethan Anesthesia Alone on Non-specific Complement Fixation with Normal Rabbit Serum

Date of Operation	Date	Reaction After Anesthesia and Operation							
		Active Serum				Inactivated Serum			
		Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids of Human Heart	Serum Control
Splenectomy Nov. 7	Nov. 7	—	—	—	—	—	+	—	—
	Nov. 8	—	—	—	—	—	+	—	—
Splenectomy Nov. 7	Nov. 7	—	—	—	—	++	+++	++	—
	Nov. 9	—	—	—	—	—	—	—	—
	Nov. 13	—	—	—	—	—	++	—	—
	Nov. 16	0	0	0	0	+	+++	+++	—
	Nov. 23	0	0	0	0	+++	++	++	—
Anesthesia only Nov. 7	Nov. 7	—	—	—	—	+++	+++	+++	—
	Nov. 8	—	—	—	—	+	+	—	—
Anesthesia only Nov. 7	Nov. 7	—	—	—	—	++	++	++	—
	Nov. 9	—	—	—	—	—	+	—	—
	Nov. 13	—	—	—	—	+	+	+	—
	Nov. 16	0	0	0	0	++	++	±	—
	Nov. 19	0	0	0	0	++++	++++	+++	—

Nitrous oxid oxygen anesthesia has no appreciable influence on the serum reactions of normal rabbits.

Splenectomy alone has probably no influence upon the property in normal rabbit and dog sera of fixing or absorbing complement with various non-specific lipoidal and bacterial antigens, the effects being in larger degree attributable to the anesthetic; the changes observed in dogs following splenectomy under ether were somewhat more profound than those in rabbits.

STUDIES IN NON-SPECIFIC COMPLEMENT FIXATION *

IV. THE RELATION OF SERUM LIPOIDS AND PROTEINS TO NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT AND DOG SERA

J O H N   A.   K O L M E R

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Since ether and chloroform during anesthesia have been found to influence the substances in normal rabbit and dog sera responsible for absorbing complement in a non-specific manner,[1] probably through some influence on the serum lipoids, we have continued this investigation to determine more definitely the relation of these lipoids to the process by extractions of serum with lipoid solvents, as ether and chloroform, and by feeding and immunizing experiments with various lipoids.

Landsteiner and v. Eisler[2] found that blood corpuscles and serum contained lipoids that exerted antihemolytic action against serum hemolysins. They believed the action of these lipoids to be anti-amboceptoric, in the sense that they constitute the receptors, which in the intact corpuscles anchor the amboceptors. Bang and Forssmann[3] extracted ox corpuscles with ether and obtained hemolytic and antihemolytic substances, the latter being acetone-soluble and the insoluble residue containing the former. Noguchi[4] found the thermostabil anticomplementary principles of the blood to be closely identified with the serum lipoids, and by means of ether extracted a soluble fraction which was markedly antilytic for the susceptible blood corpuscles of different animals. A salt solution of this substance, to which Noguchi applied the name "protectin," was found to resist temperatures as high as 90 C. Zinsser and Johnson,[5] in a study of heat-sensitive, or thermolabil, anticomplementary bodies in human serum, extracted serum with ether, but were unable to extract the anticomplementary substances in this manner. On the other hand, according to them, the thermolabil body was removed by precipitation of the globulins.

Since in rabbit and dog sera the property of absorbing complement with various lipoidal and bacterial antigens may be due to sub-

[1] Jour. Infect. Dis., 1916, 18, p. 32.
[2] Wien. klin. Wchnschr., 1904, 27, p. 676. Centralbl. f. Bakteriol., I, O., 1905, 39, p. 309.
[3] Centralbl. f. Bakteriol., I. O., 1905, 40, p. 150.
[4] Jour. Exper. Med., 1906, 8, p. 726.
[5] Ibid., 1911, 13, p. 31.

stances closely allied to those regarded as antihemolytic, we have studied the serum lipoids and proteins with the object of determining which contained the antilytic and complement-absorbing substances, the result of our work strongly indicating the more important role of lipoids in these processes.

I

The Relation of Serum Lipoids to the Antilytic and Complement-Fixing Powers of Normal Rabbit and Dog Sera

Briefly, the plan of study included antilytic and Wassermann tests with rabbit and dog sera in a fresh and active state, and after heating at 56 C. for half an hour, before and after extraction with ether and chloroform. Other lipoid solvents, as alcohol, acetone, and benzol, were employed, but these caused such pronounced physical changes of the serum that most of our work was conducted with ether as the solvent.

Technic

Sera were extracted by diluting with 4 parts of sterile salt solution, adding 5 parts of ether, and shaking in a mechanical shaker for 2 hours. The mixtures were placed in a refrigerator over night; then the supernatant ether was pipetted off, and the serum filtered repeatedly through fat-free paper until all odor of ether had been removed. Each cubic centimeter of the serum residue represented 0.2 c.c. of the original amount, and this dose and fractions of it were regularly used in the antilytic and complement-fixation tests to determine comparative values. Sera extracted with chloroform, acetone, etc., required centrifugation, after which the diluted serum could be removed and filtered.

The Wassermann tests were conducted with 5 different antigens: an alcoholic extract of heart re-enforced with cholesterin; an alcoholic extract of syphilitic liver; an extract of acetone-insoluble lipoids of heart; an emulsion of staphylococci (human) and colon bacilli (human). The lipoidal tissue extracts were employed in doses varying from 6 to 12 times less than their anticomplementary doses, according to the extract and titrations; the bacterial antigens were titrated each day and used in amounts equal to one-fourth of their anticomplementary doses.

Complement was furnished by the mixed sera of at least 2 guinea-pigs and used in constant dosage of 0.05 c.c. (1 c.c. of 1 : 20 dilution). Antisheep amboceptor was titrated against this dose of complement and 1 c.c. of a 2.5% suspension of washed cells and used in the titrations, and in the antilytic, and Wassermann tests in an amount equal to 2 hemolytic units.

Antilytic tests were conducted by mixing serum and complement and incubating at 37 C. for an hour; then 1 c.c. of corpuscle suspension and 2 units of hemolysin were added; after re-incubation for an hour the results were read.

Complement-fixation tests were conducted by incubating together for one hour serum, antigen, and complement; corpuscles and 2 units of hemolysin were then added and re-incubation continued for 1 hour, after which the results were read.

ANTILYTIC ACTIVITY OF EXTRACTED SERA

The antilytic activity of dog and rabbit sera was determined with sera in a fresh or active condition, and again after heating at 56 C. for 30 minutes. Portions of each of these sera were extracted with ether after the method described, and the antilytic tests repeated with the unheated and heated serum residues. Portions of sera were also heated and then extracted, and antilytic tests conducted with the serum residues before and after reheating at 56 C.

TABLE 1
Antilytic Activity of Plain and Extracted Dog Serum (Dog 538)

Amt. c.c.	Fresh Serum	Heated Serum	Extracted Serum, Not Heated	Extracted Serum, Heated	Heated Serum, Extracted, Not Reheated	Heated Serum, Extracted, Reheated
0.1	Complete hemolysis (not anti-lytic)	Complete hemolysis (not anti-lytic)	No hemoly-sis (strongly antilytic)	Complete hemolysis (not anti-lytic)	No hemoly-sis (strongly antilytic)	Complete hemolysis (not anti-lytic)
0.2	Complete hemolysis (not anti-lytic)	Marked hemolysis (slightly antilytic)	No hemoly-sis (strongly antilytic)	Complete hemolysis (not anti-lytic)	No hemoly-sis (strongly antilytic)	Complete hemolysis (not anti-lytic)
0.4	Complete hemolysis (not anti-lytic)	No hemoly-sis (strongly antilytic)	No hemoly-sis (strongly antilytic)	Marked hemolysis (slightly antilytic)	No hemoly-sis (strongly antilytic)	Marked hemolysis (slightly antilytic)
0.6	Complete hemolysis (not anti-lytic)	No hemoly-sis (strongly antilytic)	No hemoly-sis (strongly antilytic)	Marked hemolysis (slightly antilytic)	No hemoly-sis (strongly antilytic)	Slight hemolysis (moderately antilytic)
0.8	Complete hemolysis (not anti-lytic)	No hemoly-sis (strongly antilytic)	No hemoly-sis (strongly antilytic)	Slight hemolysis (moderately antilytic)	No hemoly-sis (strongly antilytic)	Slight hemolysis (moderately antilytic)
1.0	Complete hemolysis (not anti-lytic)	No hemoly-sis (strongly antilytic)	No hemoly-sis (strongly antilytic)	No hemoly-sis (strongly antilytic)	No hemoly-sis (strongly antilytic)	No hemoly-sis (strongly antilytic)

This plan of study was rendered necessary by the fact that antilytic and complement-absorbing powers are increased in rabbit and dog sera heated at temperatures between 55 and 60 C. for half an hour. This constitutes an important characteristic feature of these sera and one to which we have endeavored to draw particular attention both from practical and theoretical standpoints.

The results with a dog and a rabbit serum are shown in Tables 1 and 2; these are types of the results observed with similar titrations of 5 different dog, and 4 different rabbit sera.

Titrations of 3 dog, and 2 rabbit sera, after chloroform extractions, have yielded results similar to those obtained after extraction with ether. These results may be summarized as follows:

1. Heating normal rabbit and dog sera greatly increases the antilytic activity.

2. Sera extracted with ether and chloroform and tested at once are highly antilytic.

3. Sera extracted with ether and chloroform and then heated at 56 C. for 30 minutes usually possess a diminished antilytic activity.

TABLE 2

Antilytic Activity of Plain and Extracted Rabbit Serum (Rabbit 65)

Amt. c.c.	Fresh Serum	Heated Serum	Extracted Serum, Not Heated	Extracted Serum, Heated	Heated Serum, Extracted, Not Reheated	Heated Serum, Extracted, Reheated
0.1	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	No hemolysis (strongly antilytic)	Complete hemolysis (not antilytic)	No hemolysis (strongly antilytic)	Complete hemolysis (not antilytic)
0.2	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	No hemolysis (strongly antilytic)	Complete hemolysis (not antilytic)	No hemolysis (strongly antilytic)	Complete hemolysis (not antilytic)
0.4	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	No hemolysis (strongly antilytic)	Complete hemolysis (not antilytic)	No hemolysis (strongly antilytic)	Complete hemolysis (not antilytic)
0.6	Complete hemolysis (not antilytic)	Marked hemolysis (slightly antilytic)	No hemolysis (strongly antilytic)	Marked hemolysis (slightly antilytic)	No hemolysis (strongly antilytic)	Marked hemolysis (slightly antilytic)
0.8	Complete hemolysis (not antilytic)	Slight hemolysis (moderately antilytic)	No hemolysis (strongly antilytic)	No hemolysis (strongly antilytic)	No hemolysis (strongly antilytic)	Marked hemolysis (slightly antilytic)
1.0	Complete hemolysis (not antilytic)	No hemolysis (strongly antilytic)	No hemolysis (strongly antilytic)	No hemolysis (strongly antilytic)	No hemolysis (strongly antilytic)	No hemolysis (strongly antilytic)

4. Sera first heated and then extracted with ether and chloroform are markedly antilytic; when the extracted sera are again heated, the antilytic activity is about the same as in fresh sera after extraction and heating.

5. According to the method of extraction employed. ether and chloroform extract from dog and rabbit sera a portion, but not all, of the antilytic substances, probably lipoidal in nature. Since extraction of fresh and that of heated sera yielded similar results, it is probable that the lipoid substances are unchanged by heating, in so far as

solubility in ether and chloroform are concerned, altho heating a serum results in increasing its antilytic activity either by changing the nature of the lipoidal or protein constituents or by the liberation of the anti-lysin from a combination with other bodies in normal serum.

COMPLEMENT-FIXATION TESTS WITH EXTRACTED SERA

Similar procedures were carried out with normal dog and rabbit sera, except that the tests were conducted with various antigens in place of antilysin titrations. In a number of instances both antilysin and complement-fixation tests were conducted with the same sera for purposes of comparison. The results are shown in Tables 3 and 4.

These results may be summarized as follows:

1. Fresh sera that are extracted with ether or chloroform without subsequent heating are so highly antilytic that complement-fixation reactions can not be conducted with them. This observation, which has been previously described in this paper, shows that extraction of sera with these substances increases the antilysin content of sera either by releasing the antilysin from combination with other serum constituents or by some alteration of the latter. These apparently newly formed antilysins are thermolabil; heating extracted sera removes a large portion of them.

2. The results obtained with chloroform extraction were similar to those observed with extraction by ether, except that chloroform tends to remove the antilysin either less completely or else alters the protein or other serum constituents in such manner as to increase antilytic activity.

3. Extraction of normal rabbit and dog sera with ether tends to remove a large portion of the serum constituents that are responsible for the complement fixation with non-specific lipoidal and bacterial antigens. At one time we thought that this could be developed into a practical method for overcoming the difficulty of non-specific complement fixation with these sera, but the results were somewhat too irregular, and furthermore, as will be shown later, the process of extraction tends to remove or destroy to some extent the specific amboceptors in serum.

4. Sera that are highly antilytic, as some of those shown in Table 4 (Dogs 1 and 2; Mules 1, 3, and 4), are slightly or not at all affected by extraction with ether. The same was true after extraction with chloroform, and it is probable that not all antilysins are lipoidal and

TABLE 3

RESULTS OF COMPLEMENT-FIXATION TESTS WITH ANIMAL SERA BEFORE AND AFTER EXTRACTION WITH ETHER AND HEATED AT 56 C. FOR 30 MINUTES

Amount of Serum in c.c.	Plain Heated Serum						Serum Extracted and Heated					
	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	Colon Bacilli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	Colon Bacilli	Serum Control
A. Normal Dog Serum												
0.01	—	—	—	—	—	—	—	—	—	—	—	—
0.05	—	—	—	+	+	—	—	—	—	—	—	—
0.1	++	+	—	++	++	—	—	—	—	—	—	—
0.15	++	++	+	++++	++++	—	—	—	—	—	—	—
0.2	+++	++	+	++++	++++	—	—	—	—	—	—	—
0.3	+++	++	++	++++	++++	±	±+	—	—	+	+	—
B. Normal Rabbit Serum												
0.01	—	—	—	++	++	—	—	—	—	—	—	—
0.05	—	—	—	++++	++++	—	—	—	—	—	—	—
0.1	++	+	+	++++	++++	—	—	—	—	—	—	—
0.15	+++	++	++	++++	++++	—	—	—	—	—	—	—
0.2	+++	++	++	++++	++++	—	—	—	—	++	++	—
0.3	++++	++	++	++++	++++	±	±+	±	—	+++	+++	—

KEY TO TABLES 3, 4, 5, 6, 7, 8, 9 and 11

++++ = complete inhibition of hemolysis (strongly positive).
+++ = 75% inhibition (moderately positive).
++ = 50% inhibition (weakly positive).
+ = 25% inhibition (weakly positive).
± = less than 25% inhibition (doubtfully positive).
— = complete hemolysis (negative).

TABLE 4

RESULTS OF COMPLEMENT-FIXATION TESTS WITH DOG, RABBIT, MULE, AND HORSE SERA BEFORE AND AFTER EXTRACTION WITH ETHER AND HEATED AT 56 C. FOR 30 MINUTES

Serum	Heated Serum (0.2 c.c.)						Serum Extracted and Heated (0.2 c.c.)					
	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	Colon Bacilli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	Colon Bacilli	Serum Control
Dog 1												
Dog 2												
Dog 3												
Dog 4												
Dog 5												
Dog 66												
Rabbit 1												
Rabbit 2												
Rabbit 3												
Rabbit 4												
Rabbit 5												
Rabbit 6												
Mule 1												
Mule 2												
Mule 3												
Mule 4												
Horse 1												
Horse 2												

soluble in these solvents, but that antilysins may be of other structure and identified with the protein constituents.

5. As shown in Table 5, sera may possess very slight or no antilytic power and yet absorb complement to a well-marked degree in the presence of a lipoidal antigen (cholesterinized alcoholic extract of heart) and an indifferent bacterial antigen (staphylococci from human lesions). As repeatedly stated, this is the most important factor in complement fixation with rabbit, dog, and mule sera, as the serum control tubes containing the maximal amount of serum may show complete hemolysis, and yet this amount of serum or less is capable of absorbing or fixing complement in a non-specific manner in the presence of various indifferent antigens.

TABLE 5

Results of Antilytic and Complement-fixation Tests with Normal Dog and Rabbit Sera Heated at 56 C. for 30 Minutes

Amount of Serum in c.c.	Dog 10			Dog 15			Rabbit 20		
	Antilytic Test	Complement Fixation		Antilytic Test	Complement Fixation		Antilytic Test	Complement Fixation	
		Cholesterinized Alcoholic Extract of Heart	Staphylococci		Cholesterinized Alcoholic Extract of Heart	Staphylococci		Cholesterinized Alcoholic Extract of Heart	Staphylococci
0.01	—	—	—	—	—	—	—	—	+
0.05	—	—	+	—	—	—	—	±	+++
0.1	—	+	++	—	±	—	—	+	++++
0.15	—	++	+++	—	+	—	—	++	++++
0.2	—	++	++++	—	++	—	—	+++	++++
0.3	±	++	++++	—	++	±	±	++++	++++

In this table the — and ± signs in the antilytic tests indicate "complete hemolysis" and "almost complete hemolysis," respectively.

6. As shown in Table 6 extraction of the sera of syphilitics (human) with ether and chloroform tends to remove thermostabil antilytic substances (Nos. 4, 5, and 8), as shown by Noguchi, and also to some extent the Wassermann antibody or reagin (Nos. 3, 6, and 8). This action, however, was not constant in either direction, as the antilytic power of some sera was slightly increased (Nos. 1 and 2) and the Wassermann reagin undisturbed (Nos. 1, 2, 4, and 5), as the result of extractions. When similar tests were made with the sera of glanderous mules with specific glanders antigen, the same tendency toward removal of specific amboceptors was noted. On the other hand, it may be that the removal of antilysin was responsible for the weaker complement fixation and that the specific amboceptors were not removed.

7. The tendency of ether and chloroform to remove in vitro the substances responsible for complement fixation with non-specific antigens is similar to the action of these substances in vivo when administered as anesthetics; for, as shown by Kolmer and Pearce, the tendency for normal rabbit and dog serum to yield these non-specific complement fixations is removed or decreased after the administration of ether and chloroform, probably as the result of a solvent action on the lipoid constituents of the serum.

TABLE 6

Results of Complement-fixation Tests with Human Wassermann-positive Sera Before and After Extraction with Ether and Chloroform and Heated at 56 C. for 30 Minutes (Dose 0.2 c.c.)

No.	Plain Heated Serum				Ether Extracted and Heated				Chloroform Extracted and Heated			
	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Serum Control	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Serum Control	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Serum Control
1	++	+	+	—	+++	++	++	+	+++	++	++	+
2	++++	++++	++++	—	++++	++++	++++	±	++++	++++	++++	+
3	++++	+++	++++	—	+	+	+	—	—	—	—	—
4	++++	++++	++++	+	++++	++++	++++	—	++++	++++	++++	—
5	++++	+	++	—	++++	+	+	—	++++	±	±	—
6	++++	+++	++++	+	++	+	+	—	0	0	0	0
7	++++	++++	++++	—	+	+	+	±	0	0	0	0
8	++++	++++	++++	+	±	±	±	—	+	+	+	—

THE FEEDING OF LIPOIDS IN RELATION TO NON-SPECIFIC COMPLEMENT FIXATION

As our previous experiments indicated the important rôle of serum lipoids as antilysins and their responsibility in some degree for non-specific complement fixation, it appeared of interest to determine whether feeding lipoids to selected animals would cause the appearance in the blood serum of antilysins, as the high antilytic and complement-absorbing power of the sera of these animals may bear some relation to the diet. (This portion of the work was conducted with the co-operation of Dr. Richard M. Pearce, of the John Herr Musser Department of Research Medicine of the University of Pennsylvania.) Rabbits the sera of which repeatedly failed to fix complement with the various lipoidal antigens, were selected for these experiments.

Three lipoids were administered: (a) cholesterin isolated from human gallstones and Merck's preparation in doses of 0.5 to 0.13 gram; (b) Merck's lecithin in dosage of 0.5 to 0.13 gram; (c) glymol (a purified paraffin oil) in dosage of 10 c.c. These lipoids were administered in capsule, and by the stomach tube, and in one series were injected in the form of an emulsion or were placed under the skin.

The antigens employed in the complement-fixation tests consisted of the 3 usual lipoidal extracts—cholesterinized heart extract, alcoholic extract of syphilitic liver, an extract of acetone-insoluble lipoids of heart muscles—and solutions of cholesterin (0.4%), lecithin (Merck), (0.5%), and glymol (10%) in absolute ethyl alcohol. These 3 preparations were diluted with normal salt solution, repeatedly titrated, and used in amounts equal to one-quarter of their antilytic dose. The lecithin solution was especially difficult to titrate because of the marked hemolytic properties of the preparation.

The complement-fixation tests were conducted with the same technic as previously described.

The results of these experiments are shown in Tables 7, 8 and 9, and may be summarized as follows:

1. The feeding of cholesterin, lecithin, and glymol to normal rabbits appears to be followed by an increased power of the serum for the absorption or inhibition of complement. This was especially evident after the feeding of lecithin and much less so with glymol.

2. This increased power of the serum for complement absorption or inhibition was in evidence practically only with heated serum. No changes were apparent when fresh, unheated serum was used.

3. The increased power of the sera for complement absorption or inhibition was apparent within 48 hours after the administration of the lipoids; after several days the sera returned to their former states.

4. The degree of complement absorption was highest with the antigen of lecithin, next highest with that of cholesterin, and least with that of glymol. Specific reactions were not observed; that is, the administration of cholesterin was followed by an increased power for the absorption of complement not only with an antigen of cholesterin, but likewise with that of lecithin. The stronger reactions with the antigens of cholesterin, lecithin, and glymol may be the result of using these in doses equal to one-fourth of their anticomplementary doses,

TABLE 7

RESULTS OF COMPLEMENT-FIXATION TESTS WITH THE SERA OF NORMAL RABBITS BEFORE AND AFTER THE ADMINISTRATION OF CHOLESTERIN

Rabbit	Cholesterin	Active Serum (0.2 c.c.)							Inactivated Serum (0.2 c.c.)						
		Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Choles-terin	Leci-thin	Gly-mol	Serum Con-trol	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Choles-terin	Leci-thin	Gly-mol	Serum Con-trol
25	11 days before	—	—	—	0	0	0	—	—	—	—	0	0	0	—
25	8 days before	—	—	—	0	0	0	—	—	—	—	0	0	0	—
25	2 days before	—	—	—	—	—	—	—	—	—	—	—	—	—	—
25	0.5 gm. cholesterin (stomach)												+		
25	2 days later	—	—	—	—	—	—	—	+	—	+	+	+ +	+	—
25	7 days later	—	—	—	—	—	—	—	+	—	—	+	+	—	—
25	0.5 gm. cholesterin (stomach)														
25	2 days later	—	—	—	0	0	0	—	+	—	—	+	+	0	—
26	11 days before	—	—	—	0	0	0	—	0	—	—	0	0	0	—
26	8 days before	—	—	—	—	—	—	—	—	—	—	—	—	—	—
26	2 days before	—	—	—	—	—	—	—	—	—	—	—	—	—	—
26	0.5 gm. cholesterin (stomach)														
26	2 days later	—	—	—	—	—	—	—	—	—	—	+ ±	+ +	—	—
26	7 days later	—	—	—	—	—	—	—	—	—	—	+	+	—	—
26	0.5 gm. cholesterin (stomach)														
26	2 days later	—	—	—	—	—	—	—	—	—	—	—	—	—	—
83	5 days before	—	—	—	—	—	—	—	—	—	—	—	—	—	—
83	2 days before	—	—	—	—	—	—	—	—	—	—	—	—	—	—
83	0.13 gm. cholesterin (stomach)														
83	1 day later	—	—	—	—	—	—	—	—	+	—	+	+	—	—
65	2 days before	—	—	—	—	—	—	—	+	+	+	+	+	+	—
65	0.13 gm. cholesterin (subcutaneously)														
65	1 day later	—	—	—	—	—	—	—	+	+	+	+	+ + + +	+	—
65	3 days later	—	—	—	—	—	—	—	+	+	+	+	+ + + +	+	—
65	4 days later	—	—	—	—	—	—	—	+	+	+	+	+ + + +	+	—

TABLE 8

RESULTS OF COMPLEMENT-FIXATION TESTS WITH THE SERA OF NORMAL RABBITS BEFORE AND AFTER THE ADMINISTRATION OF LECITHIN

Rabbit	Lecithin	Active Serum (0.2 c.c.)							Inactivated Serum (0.2 c.c.)						
		Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Cholesterin	Lecithin	Glymol	Serum Control	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	Cholesterin	Lecithin	Glymol	Serum Control
36	11 days before	—	—	—	o	o	o	—	—	—	—	o	o	o	—
36	8 days before	—	—	—	o	o	o	—	—	—	—	o	o	o	—
36	2 days before														
36	0.5 gm. lecithin (stomach)														
36	2 days later	—	—	—	—	—	—	—	+	+	+	+	+H	—	—
36	7 days later														
36	0.5 gm. lecithin (stomach)	—	—	—	o	o	o	—	—	+	+	+	+	o	o
36	2 days later	—	—	—	o	o	o	—	—	—	—	o	o	o	—
36	11 days later														
29	8 days before														
29	2 days before														
29	0.5 gm. lecithin (stomach)	—	—	—	+	+	—	—	+	+	+	+	+	—	—
29	2 days later														
29	7 days later														
29	0.5 gm. lecithin (stomach)	—	—	—	+	+	—	—	+	+	+	+	+H	—	—
29	2 days later														
68	5 days before	—	—	—	—	—	—	—	—	—	—	—	H+++	—	—
68	2 days before	—	—	—	+	+	+	—	+	+	+	++	+++	+	—
68	0.13 gm. lecithin (stomach)														
68	1 day later	—	—	—	—	—	—	—	—	—	—	—	++	+	—
68	3 days later														
68	4 days later														
68	7 days later														
68	5 days before														
69	2 days before	—	—	—	—	—	—	—	—	—	—	—	+++	—	—
69	0.13 gm. lecithin (subcutaneously)														
69	1 day later	—	—	—	—	—	—	—	—	—	—	—	+++	—	—
69	3 days later														
69	4 days later (died)	—	—	—	—	—	—	—	—	—	—	—	++	—	—

TABLE 9

Results of Complement-fixation Tests with the Sera of Normal Rabbits Before and After the Administration of Glymol

Rabbit	Glymol	Active Serum (0.2 c.c.)							Inactivated Serum (0.2 c.c.)						
		Cholesterinized Alcoholic Ext. of Heart	Alcoholic Ext. of Syphilitic Liver	Ext. Acetone Insoluble Lipoids of Heart	Cholesterin	Lecithin	Glymol	Serum Control	Cholesterinized Alcoholic Ext. of Heart	Alcoholic Ext. of Syphilitic Liver	Ext. Acetone Insoluble Lipoids of Heart	Cholesterin	Lecithin	Glymol	Serum Control
38	11 days before	—	—	—	0	0	0	—	—	—	—	0	0	0	—
38	8 days before	—	—	—	0	0	0	—	—	—	—	0	0	0	—
38	2 days before	—	—	—				—	—	—	—				—
38	10 c.c. glymol (stomach)	—	—	—				—	—	—	—				—
38	2 days later	—	—	—				—	—	—	—				—
38	7 days later	—	—	—	0	0	0	—	—	—	—	0	0	0	—
38	15 days before	—	—	—	0	0	0	—	—	—	—	0	0	0	—
38	7 days before	—	—	!				—	—	—	—				—
38	10 c.c. glymol (stomach)	—	—	—				—	+	+	—	++	+++	+	—
38	2 days later	—	—	—				—	—	—	—	!	+++	—	—
66	5 days before	—	—	—				—	—	—	—	!	+++	—	—
66	2 days before	—	—	—				—	—	—	—				—
66	5 c.c. glymol (stomach)	—	—	—				—	+	+	+	++	++++	++	—
66	1 day later	—	—	—				—	+	+	+	++	++++	++	—
66	3 days later	—	—	—				—	—	—	—		+++		—
66	4 days later	—	—	—				—	—	—	—		++		—
67	5 days before	—	—	—				—	—	—	—		+		—
67	2 days before	—	—	—				—	—	—	—		++++		—
67	5 c.c. glymol (subcutaneously)	—	—	—				—	+	+	+	+	++++	+	—
67	1 day later	—	—	—				—	—	—	—		+		—
67	3 days later (died)	—	—	—				—	—	—	—		++++		—

whereas the regular Wassermann antigens were used in doses equal to one-sixth to one-twelfth of their anticomplementary doses.

5. The subcutaneous administration of cholesterin, lecithin, and glymol was followed by slow absorption and with less influence upon the serum reactions.

Several of these sera were extracted with ether and the complement-fixation tests repeated with the antigens after heating the sera at 56 C. for half an hour. In practically all instances the complement-absorbing power of the serum was reduced, as a result, apparently, of the removal of ether-soluble substances.

These experiments indicate, therefore, that following the feeding of lipoids some absorption occurs, resulting in an increase of the complement-absorbing power of the serum and further indicating the rôle that serum lipoids play in the process.

II

The Relation of the Serum Proteins to the Antilytic and Non-specific Complement-fixation Powers of Normal Rabbit and Dog Sera

Since in our experiments the extraction of lipoids from normal rabbit and dog sera did not serve to remove all the substances responsible for the antilytic and non-specific complement-fixation reactions, it is probable that other serum constituents are also concerned. The proteins of the serum may act in this capacity, since it is well known that solutions of proteins in sufficient concentration possess antilytic or anticomplementary properties. My particular object was to ascertain, if possible, whether these properties were contained in the globulin or in the albumin fractions, or in both, particularly since, according to Zinsser and Johnson, the globulin fraction of human serum contains the thermolabil anticomplementary substances, while Noguchi has identified the thermostabil anticomplementary substances of human serum with the serum lipoids.

Two specimens each of normal rabbit and dog sera were fractionated and the antilytic and complement-fixing powers determined. Five cubic centimeters of fresh serum were diluted with 45 c.c. of sterile normal salt solution, and an equal amount (50 c.c.) of a saturated solution of ammonium sulfate was added. The precipitated globulins were removed by repeated filtration through 5 thicknesses of fine filter paper. All the precipitate on the filters was then dissolved in 50 c.c. of normal salt solution, containing 0.25% tricresol, and the solution dialyzed against running water for 72 hours in order to remove the ammonium

sulfate; practically all the globulins were removed, and, when they were redissolved in the same bulk, 1 c.c. represented very closely the globulins contained in 0.1 c.c. of undiluted serum.

TABLE 10

RESULTS OF ANTILYTIC TESTS WITH FRESH, HEATED, AND ETHER-EXTRACTED SERA OF A NORMAL DOG AND THE GLOBULIN AND ALBUMIN FRACTIONS OF THESE

Dose c.c.	Fresh Serum	Heated Serum	Ether Extracted Serum	Globulin of Fresh Serum	Globulin of Heated Serum	Globulin of Ether Extracted Serum	Filtrate of Fresh Serum	Filtrate of Heated Serum	Filtrate of Ether Extracted Serum
.05 (.005)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)
.1 (.01)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)
.2 (.02)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)
.5 (.05)	Complete hemolysis (not antilytic)	Marked hemolysis (slightly antilytic)	Complete hemolysis (not antilytic)	Marked hemolysis (slightly antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)
1.0 (0.1)	Marked hemolysis (slightly antilytic)	No hemolysis (strongly antilytic)	Complete hemolysis (not antilytic)	Slight hemolysis (moderately antilytic)	Marked hemolysis (slightly antilytic)	Complete hemolysis (not antilytic)	Marked hemolysis (slightly antilytic)	Complete hemolysis (not antilytic)	Complete hemolysis (not antilytic)
2.0 (0.2)	Slight hemolysis (moderately antilytic)	No hemolysis (strongly antilytic)	Marked hemolysis (slightly antilytic)	No hemolysis (strongly antilytic)	Marked hemolysis (slightly antilytic)	Marked hemolysis (slightly antilytic)	No hemolysis (strongly antilytic)	Marked hemolysis (slightly antilytic)	Complete hemolysis (slightly antilytic)

Tricresol to 0.25% was added to the filtrate to prevent bacterial growth during dialyzation, and the whole dialyzed against running water for 72 hours. One cubic centimeter of the filtrate represented 0.1 c.c. of undiluted serum.

After this period of dialyzation practically all the ammonium sulfate had been removed as determined by tests with barium chlorid. In both the globulin

solutions and filtrates, however, traces remained. Several solutions became cloudy and showed a small amount of precipitate. These were filtered before being used in the hemolytic tests. As all the sodium chlorid had dialyzed, each solution was made isotonic (0.85) by the addition of sodium chlorid.

Several controls composed of equal parts of ammonium sulfate and salt solution were dialyzed at the same time. This solution possesses marked antilytic properties, but after dialyzation the minute trace of the remaining salt had no effect in doses ranging from 0.05 to 2 c.c.

The antilytic titer of each whole serum (diluted 1 : 10), filtrate, and solution of globulins was determined by titrating in 6 doses; namely, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 c.c., corresponding, respectively, to 0.005, 0.01, 0.02, 0.05, 0.1, and 0.2 c.c. of undiluted serum.

Complement-fixation tests were conducted with these doses, with as antigen an alcoholic extract of beef heart re-enforced with cholesterin in dose equal to one-twelfth of its anticomplementary dose.

These tests were conducted with unheated serum, filtrates, and globulins, and again after these had been heated at 56 C. for half an hour. The antilytic and complement-fixing values were lower with the heated than with the unheated solutions, presumably on account of the removal of thermolabil anticomplementary substances which had developed during the process of dialyzation.

TABLE 11

RESULTS OF COMPLEMENT-FIXATION TESTS WITH FRESH, HEATED AND ETHER-EXTRACTED SERA OF A NORMAL DOG AND THE GLOBULIN AND ALBUMIN FRACTIONS OF THESE

Dose c.c.	Fresh Serum	Heated Serum	Ether Ex-tracted Serum	Globulin of Fresh Serum	Globulin of Heated Serum	Globulin of Ether Ex-tracted Serum	Filtrate of Fresh Serum	Filtrate of Heated Serum	Filtrate of Ether Ex-tracted Serum
.05 (.005)	—	—	—	—	—	—	—	—	—
.1 (.01)	±	+	—	—	—	—	—	—	—
.2 (.02)	++	+++	—	—	—	—	—	—	—
.5 (.05)	+++	++++	—	+	—	—	—	—	—
1.0 (0.1)	++++	++++	++	++	+	—	++	++	—
2.0 (0.2)	++++	++++	+++	++++	++	+++	++++	++	++

With each of 2 rabbit and 2 dog sera the antilytic and complement-fixing values of each of the following were determined:

1. Fresh unheated serum.

2. Serum heated at 56 C. for half an hour.

3. Serum extracted with ether, according to the method previously described, and heated.

4. The globulin and filtrate of fresh serum.

5. The globulin and filtrate of heated serum.

6. The globulin and filtrate of ether-extracted serum.

The results observed with one of the dog sera are shown in Tables 10 and 11, and these illustrate the results obtained with the other three sera. They may be summarized as follows:

1. Heating the sera at 56 C. for half an hour increases the antilytic and complement-fixing powers.

2. Extraction with ether serves to remove a large percentage of the antilytic and complement-fixing substances.

3. Both the globulin and albumin (filtrate) portions of rabbit and dog sera possess antilytic and complement-fixing properties. In general, the globulin fractions show these properties to a slightly greater degree.

4. The antilysins contained in the globulin and albumin (filtrate) fractions are thermostabil.

5. According to these results I must conclude that the protein constituents of serum represent a portion of the antilytic and complement-fixing substances in normal rabbit and dog sera and that both the globulin and albumin fractions are concerned, the former to a slightly greater degree.

ANTILYSINS NOT DIALYZABLE

Two rabbit and two dog sera were diluted 1 : 10 with normal salt solution, 0.25% tricresol added, and the whole dialyzed against running water for 72 hours. Antilytic and complement-fixation tests with these sera compared with similar tests with non-dialyzed sera showed that none of the antilytic substances had been removed; indeed, the dialyzed sera were slightly more antilytic.

CONCLUSIONS

Both the serum lipoids and proteins are concerned in the antilytic and non-specific complement-fixation reactions with normal rabbit and dog sera.

The rôle of the serum lipoids in these processes is indicated in the observation that extraction of suitable sera with ether and chloroform usually diminished the antilytic and complement-fixing powers of a serum, whereas the enteral and parenteral administration of lipoids increased the antilytic and complement-fixing powers.

Sera that are extracted with ether are rendered primarily more antilytic; after heating an extracted serum the antilytic titer is generally reduced, as compared with the titer of plain heated serum.

Both the globulin and albumin (filtrate) fractions of normal rabbit and dog sera possess thermostabil antilytic and complement-fixing properties, usually the former to a slightly greater degree.

The antilytic and complement-fixing substances of normal rabbit and dog sera are not dialyzable.

STUDIES IN NON-SPECIFIC COMPLEMENT FIXATION *

V. THE EFFECT OF HEAT ON NORMAL RABBIT AND DOG SERA IN RELATION TO ANTILYTIC AND NON-SPECIFIC COMPLEMENT-FIXATION REACTIONS

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Having considered in previous articles[1] the property of normal rabbit and dog sera of yielding non-specific complement-fixation with various lipoidal and bacterial antigens, and the influence of anesthetics and splenectomy[2] upon this property, and the relation of serum lipoids to the process,[3] in the present article we shall study further the nature and mechanism of the process, and the influence of certain factors upon it, and we shall consider methods for lessening or abolishing its effects in complement-fixation tests with these sera for specific immune bodies.

ANTIHEMOLYTIC POWER OF BLOOD SERUM IN RELATION TO NON-SPECIFIC COMPLEMENT FIXATION

Of primary importance in this connection is a consideration of the anticomplementary or antihemolytic properties of normal blood serum in relation to non-specific complement fixation on the basis that these substances are principally involved, and that in these reactions with normal rabbit and dog sera we have but a summation of the anti-complementary effects of antigen and some substance in the serum.

It is well known that normal human serum, as well as that of animals, may develop antihemolytic properties within a short time after bleeding, even under sterile conditions. Usually the influence of these antilytic substances may be removed by heating the serum at 55 C. for one-half hour, the antilysins in such case being known as thermolabil antilytic or anticomplementary substances; older sera under sterile conditions, sera containing large amounts of dissolved hemoglobin, or comparatively fresh sera infected with various micro-

organisms, may develop antilytic properties that cannot be removed by this process of heating, and to these substances has been applied the term thermostabil anticomplementary substances. Both these substances are probably of the same general structure and nature, and their effects quantitative rather than qualitative. They are of the greatest importance, however, in all complement-fixation tests, for a serum containing them is capable of absorbing or fixing varying amounts of hemolytic complement in a non-specific manner with a tissue or bacterial antigen.

While fresh, normal human serum alone may contain practically none of these antilytic substances, and while a lipoidal extract may be used as antigen in an amount 10 or 20 times less than its antilytic or anticomplementary dose, yet a combination of serum and extract in a complement-fixation test may show appreciable, or even well-marked, degrees of complement fixation in from 1 to 7% of sera. Noguchi has studied these reactions with particular care and to them has given the name of proteotropic, or false, complement-fixations.

These proteotropic reactions are non-specific, in that they may occur with perfectly fresh serum of a normal individual and with various lipoidal extracts as antigens. Noguchi has observed, however, that they are more likely to occur with crude alcoholic extracts of organs as antigen than with purified extracts, as the acetone-insoluble lipoids fraction. Our studies with fresh, normal rabbit serum and dog serum have given us similar results, but in a larger percentage of sera, especially with dog serum. In other words, the occurrence of complement fixation in a mixture of fresh and active, normal rabbit or dog serum with a lipoidal antigen is similar in several respects to the proteotropic, or false, reaction sometimes found with normal human serum. Likewise, our figures show that crude tissue extracts give a higher percentage of non-specific reactions than extracts freed of various extractives, fatty acids, etc., the extract of acetone-insoluble lipoids yielding the lowest percentage of false, or non-specific, reactions, and those of alcoholic extracts of heart and the same re-enforced with cholesterin, the highest percentage of positive reactions.

The remarkable difference in this connection, however, is that, while the antilytic properties of fresh human serum may be removed or greatly lessened by heating at 56 C. for half an hour, with rabbit, dog, and mule sera the reverse is noted, these sera becoming more antilytic when heated, and yielding higher percentages of pseudo-com-

plement-fixation.   Human serum may develop antilytic properties as
the result of heating, but this action is not in evidence in complement-
fixation tests as ordinarily conducted; whereas the important practical
significance of the behavior of dog and rabbit sera in complement-
fixation tests is noted when these sera are inactivated by heating at
56 C. for one-half hour and used in complement-fixation tests with
various and indifferent tissue extracts and bacterial emulsions as
antigens.   Then well-marked complement fixation occurs, while in
those test tubes containing no antigen, as the serum control tubes,
hemolysis is complete.

It is now generally accepted that these serum antilysins exert
their antilytic activity by some action upon the complements, and
hence they are more generally known as anticomplementary sub-
stances.   Their relation to the substances causing non-specific com-
plement fixation is so intimate that in this work it was necessary to
study the subject from both standpoints.

Technic

The complement-fixation tests were conducted with 3 lipoidal extracts.
namely, an alcoholic extract of heart re-enforced with cholesterin, an alcoholic
extract of syphilitic liver, and an extract of acetone insoluble lipoids from beef
heart; also with 3 bacterial antigens of staphylococci, colon bacilli (human),
and typhoid bacilli, described in previous papers.

The lipoidal extracts were used in the same doses as in our regular Wasser-
mann reactions, these amounts being 6 to 12 times less than the antihemolytic
doses.   The bacterial antigens were titrated before each experiment and used in
amounts equal to one-quarter of their antihemolytic doses.

Complement was furnished by the mixed sera of 2 or more guinea-pigs and
used in a constant dose of 1 c.c. of a 1:20 dilution (0.05 c.c. undiluted serum).
Antisheep hemolysin (rabbit) was titrated each day against this constant dose
of complement and 1 c.c. of a 2.5% suspension of washed sheep corpuscles, and
used in amounts equal to 2 hemolytic units in the antigen titrations and com-
plement-fixation tests.   Other hemolysins, as antihuman (rabbit) and antiox
(rabbit), were similarly titrated against their respective cells, and used in
amounts equal to double their hemolytic units.

When sera were heated at a temperature of 70 C. and higher, they were first
diluted with 4 parts of normal salt solution.

Tests for the antihemolytic action of serum were conducted by incubating
heated serum and complement for 1 hour, then adding the cells and 2 units of
hemolysin, and re-incubating for 1 hour, after which the results were read.

Complement-fixation tests were conducted by incubating antigen, serum,
and complement for an hour, then adding the cells and 2 units of hemolysin,
and re-incubating for 1 hour, after which the results were read.

The doses of complement and hemolysin employed are very impor-
tant in relation to the results.   The employment of the amount of

complement serum mentioned with 1, instead of 2, hemolytic units of hemolysin increases the percentages of antihemolytic and complement-fixation reactions and conversely an excess of either complement or hemolysin or both over the amounts mentioned tends to decrease the percentages of these reactions.

THE RELATION OF HEAT TO THE ANTIHEMOLYTIC AND NON-SPECIFIC COMPLEMENT-FIXATION PROPERTIES OF SERUM

Heat bears a very important relation to the antihemolytic property of serum and its facility for absorbing complement in a non-specific manner. While the phenomenon may occur with perfectly fresh and unheated rabbit and dog sera, it is greatly intensified as the result of heating the sera at a temperature above 60 C., whereas at higher temperatures the property is less in evidence and disappears.

Of considerable importance in this connection are the observations of Camus and Gley,[4] Müller,[5] Ehrlich and Sachs,[6] Sachs,[7] Bordet and Gay,[8] Sachs,[9] Noguchi,[10] and others, showing that human and animal sera develop antihemolytic properties as the result of heating. Ehrlich and Sachs have attributed this action to the production of complementoids, and we have made studies in this phase, to be discussed later in this paper. Noguchi found that the antihemolytic action of most sera appears when the temperature is raised above 50 C., and he extracted an antilytic principle with ether, called "protectin," that was found capable of withstanding temperatures as high as 90 C., and capable of inhibiting serum hemolysis directly by neutralizing complement, and indirectly after absorption by corpuscles by increasing their resistance. Zinsser and Johnson[11] have studied particularly the thermolabil antylsins of human serum which may be removed or rendered inactive by heating serum to 56 C. Karsner and Pearce[12] have noted the antihemolytic action of dog serum.

With the hemolytic system employed we have occasionally met with perfectly fresh dog serum that was antihemolytic in doses of 0.1 to 0.2 c.c., but the percentage is considerably increased by heating the

[4] Compt. rend. Soc. de biol., 1901, 53, p. 732.
[5] Centralbl. f. Bakteriol., 1901, 29, p. 860.
[6] Berl. klin. Wchnschr., 1902, 39, p. 492.
[7] Ibid., p. 216; Centralbl. f. Bakteriol., I, O., 1905, 40, p. 125.
[8] Ann. de l'Inst. Pasteur., 1906, 20, p. 467.
[9] Deutsch. med. Wchnschr., 1905, 31, p. 705.
[10] Jour. Exper. Med., 1906, 8, p. 726.
[11] Ibid., 1911, 13, p. 31.
[12] Ibid., 1912, 16, p. 769.

sera. Fresh serum may be antihemolytic and lose this property as a result of heating to 56 C. for one-half hour, but, as previously stated, a more significant change is the acquisition of antihemolytic activity during the process of heating. This is shown in Table 1, where fresh dog serum, free of antihemolytic activity until heated, at 56 C. acquires antihemolytic activity after an exposure of 20 to 30 minutes. Prolonged exposure to an hour and a half does not materially influence the antihemolytic substances as do higher temperatures in shorter intervals of time.

TABLE 1

THE ANTIHEMOLYTIC ACTION OF DOG AND RABBIT SERA HEATED AT 56 C. FOR VARYING INTERVALS OF TIME [DOSE 0.2 C.C. TWO UNITS OF ANTISHEEP (RABBIT) HEMOLYSIN]

Exposure in Minutes	Dog Serum 6	Dog Serum 17	Dog Serum 9	Dog Serum 30	Rabbit Serum 12	Rabbit Serum 28	Rabbit Serum 42
Fresh..........	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Moderate hemolysis (slightly antilytic)
10..............	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
20..............	Complete hemolysis	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
30..............	Moderate hemolysis (slightly antilytic)	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
45..............	Moderate hemolysis (slightly antilytic)	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Moderate hemolysis (slightly antilytic)	Complete hemolysis
60..............	Slight hemolysis (markedly antilytic)	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis
90..............	Slight hemolysis (markedly antilytic)	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis

As shown in Table 2, the antihemolytic action of heated dog serum is most apparent in doses exceeding 0.1 c.c.; with the largest amounts, as 0.8 to 1.0 c.c., sufficient normal or native antisheep hemolysin is contained in the serum to counterbalance a part of the antihemolytic action of the serum, so that hemolysis is again more complete.

As shown in Table 3, the antihemolytic activity of dog serum becomes apparent after the serum has been heated at 55 C.; at a temperature of 70 C. and higher this activity is lost—a result probably, as Noguchi has pointed out, of the liberation from the serum

of a second group of principles in themselves hemolytic directly, or indirectly by increasing the power of serum hemolysins.

This antihemolytic activity of heated serum was found when anti-human (rabbit) and antiox (rabbit) hemolysins were employed, and heated dog serum neutralizes the action of fresh serum. This antihemolytic action, therefore, is non-specific, and, as shown by Noguchi, is probably antagonistic to the serum complements.

The antihemolytic action of dog serum is rather variable, as shown in the selected examples in these tables. With the technic described

TABLE 2

The Antihemolytic Action of Dog and Rabbit Sera Heated at 56 C. for 30 Minutes
[Two Units of Antisheep (Rabbit) Hemolysin]

Amount of Serum in c.c.	Dog 535	Dog 538	Dog 548	Rabbit 65	Rabbit 40	Rabbit 82
0.1.............	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.2.............	Complete hemolysis	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.4.............	Complete hemolysis	None	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.6.............	Complete hemolysis	None	Moderate hemolysis (slightly antilytic)	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis
0.8.............	Complete hemolysis	None	Slight hemolysis (markedly antilytic)	Slight hemolysis (markedly antilytic)	Complete hemolysis	Moderate hemolysis (slightly antilytic)
1.0.............	Complete hemolysis	None	Slight hemolysis (markedly antilytic)	None	Complete hemolysis	Moderate hemolysis (slightly) antilytic)

we have occasionally found fresh dog sera in some degree antihemolytic in dosage of 0.1 to 0.2 c.c.; with sera heated at 56 C. for one-half hour the percentage found to be antihemolytic in these amounts is much increased. In complement-fixation tests with dog serum this antihemolytic activity is likely to prove disturbing and should be remembered.

Rabbit serum is much less likely to show antihemolytic activity. We have never found a perfectly fresh rabbit serum antihemolytic in doses under 0.4 c.c., unless the serum was deeply stained with hemoglobin and had been secured more than 24 hours before being tested.

Likewise, when heated, rabbit serum is much less likely than dog serum to develop antihemolytic properties. As shown in the tables, composed of selected examples from a large number of tests, a serum may develop antihemolytic properties after an exposure of 30 minutes or more at a temperature between 50 and 60 C. or higher for the same interval of time. In complement-fixation tests with fresh rabbit serum, heated at 55 C. for one-half hour and used in amounts of 0.1 to 0.2 c.c. with the hemolytic system described, we have rarely found

TABLE 3

The Antihemolytic Action of Dog and Rabbit Sera After Heating at Varying Degrees Centigrade for 30 Minutes [Dose 0.2 c.c. Two Units of Antisheep (Rabbit) Hemolysin]

Temperature C.	Dog	Dog	Dog	Dog	Rabbit	Rabbit
Fresh...........	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Moderate hemolysis (slightly hemolytic)
45..............	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
50..............	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
55..............	Complete hemolysis	Complete hemolysis	Moderate hemolysis (slightly hemolytic)	Complete hemolysis	Complete hemolysis	Moderate hemolysis (slightly hemolytic)
60..............	Moderate hemolysis (slightly hemolytic)	Complete hemolysis	Slight hemolysis (markedly antilytic)	Moderate hemolysis (slightly hemolytic)	Complete hemolysis	Complete hemolysis
65..............	Moderate hemolysis (slightly hemolytic)	Complete hemolysis	Slight hemolysis (markedly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis
70..............	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
75..............	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
80..............	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis

any interference with hemolysis in the serum control tubes. This observation has been emphasized in our previous papers on complement-fixation studies with rabbit serum.

In these respects rabbit serum is more like human serum; it is highly probable, however, that the antihemolytic substances so frequently present in dog serum and much less frequently found in rabbit serum are identical and that the differences are quantitative rather than qualitative.

As stated in previous papers, fresh, active, normal dog and rabbit sera occasionally absorb or fix complement with lipoidal and various bacterial antigens. This property is greatly increased by heating, and, as a general rule, develops at a lower temperature and with a shorter exposure than the antihemolytic activity.

The question naturally arises whether apparent complement fixation by normal dog and rabbit sera with various antigens is actually due to the influence of antihemolytic substances of the serum plus the antihemolytic action of the extracts used as antigens. If this were true, the results could be ascribed to the destruction or inactivation of complement rather than to a true absorption of complement, according to our present conception of the phenomenon of complement fixation. Are the antihemolytic and hypothetical complement-fixing bodies developed and destroyed by the same temperature, or may a serum be free of antihemolytic activity and yet yield positive complement-fixation?

As stated in this paper and emphasized in our previous communications on non-specific complement fixation by normal rabbit and dog sera, the phenomenon of complement fixation was studied with the same technic as employed in our regular Wassermann tests, in which the antihemolytic action of each serum was always controlled in the serum control tube containing the maximal dose of serum and the antigens were always carefully titrated and used in amounts at least from 4 to 12 times less than their antihemolytic doses. With ready and complete hemolysis of the serum and antigen controls, showing that the antihemolytic substances of the sera and antigens were not appreciably in evidence, with complete or well-marked inhibition of hemolysis in mixtures of sera and antigens, the suspicion was strong that complement was inactivated or absorbed in some manner rather than that the absence of hemolysis was due to the direct destructive action of serum and antigen upon complement.

As shown in Table 4, fresh, normal dog and rabbit sera may show well-marked inhibition of hemolysis with bacterial and lipoidal extracts, but, when this does not occur, heating the sera at temperatures as low as 40 to 45 C. for one-half hour increases the complement-inhibiting property. In this manner the apparent complement-fixing power of a serum is developed at a lower temperature than the antihemolytic power of the serum alone, altho the complement-inhibiting power of a serum is best developed by exposure to higher temperatures, as 55-60

TABLE 4

THE COMPLEMENT-FIXING POWER OF FRESH DOG AND RABBIT SERA HEATED AT VARYING TEMPERATURES FOR 30 MINUTES

Dog Sera

Temperature, °C.	Dog Serum 539					Dog Serum 440					Dog Serum 329				
	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control
Fresh	—	—	—	—	—	—	—	—	—	—					—
40	—	—	+++	+++	—	—	—	+++	+++	—			+++	+++	—
45	—	—	+++	+++	—	—	—	+++	+++	—			+++	+++	—
50	+++	+++	++++	++++	—	±	±	++++	++++	—			++++	++++	+|±
55	+++	+++	++++	++++	—	±	±	++++	++++	—			++++	++++	+
60	+++	+++	++++	++++	—	++	++	++++	++++	—			++++	++++	—
62	+±	+±	+++	+++	+	+	+±	+++	+++	—			+++	+++	—
65	—	—	+	++	—	—	—	+	+	—			—	—	—
70	—	—	—	—	—	—	—	—	—	—			—	—	—
80	—	—	—	—	—	—	—	—	—	—			—	—	—

Rabbit Sera

Temperature, °C.	Rabbit Serum 23					Rabbit Serum 24					Rabbit Serum 65				
	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control
Fresh	—	—	—	—	—	—	—	—	—	—			—	—	—
40	—	—	+++	+++	—	—	—	++	++	—	++	—	++	++	—
45	±	±	+++	+++	—	++	++	++	++	—	+++	+|±	+++	+++	—
50	+++	+++	++++	++++	—	+++	+++	+++	+++	—	++++	++++	++++	++++	—
55	+++	+++	++++	++++	—	+++	+++	+++	+++	—	++++	++++	++++	++++	—
60	++	++	+++	+++	—	+±	+|±	++	++	—	++	++	+++	+++	—
62	+	—	+	+	—	+|±	+|±	++	++	—	++	++	—	—	—
65	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
70	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Key to Tables 4, 5, 6, 8, 10, and 11

++++ = complete inhibition of hemolysis (strongly positive).
+++ = 75% inhibition of hemolysis (moderately positive).
++ = 50% inhibition of hemolysis (weakly positive).
+ = 25% inhibition of hemolysis (very weakly positive).
± = delayed hemolysis or doubtful reaction).
— = complete hemolysis (negative reaction).

C., just as the serum antihemolytic substances are best developed at this temperature.

We have tested in this manner 12 dog and 6 rabbit sera, those shown in the tables being selected to show the irregularities and wide limits that were found. At 62 C. many sera, not all, lose their power of inhibiting complement activity with lipoidal, but not with bacterial, antigens; 65 C. exerts a greater influence, while practically all sera lose this property with all antigens when heated at 70 C. for one-half hour. As previously stated, the antihemolytic activity of a serum alone was likewise in our experiments invariably extinguished at this temperature.

As shown in Table 5, the complement-inhibiting power of dog and rabbit sera with lipoidal antigens became evident after an exposure of 10 or 20 minutes at 56 C. and much stronger after 30 and 45 minutes, while more prolonged exposure (1½ hours) did not tend to decrease this action. The results shown in this table are with sera selected among similar tests with 15 different dog, and 6 different rabbit sera, and they represent the results generally observed. As pointed out in our previous papers, rabbit sera show this complement-inhibiting power in the presence of various antigens to a much less degree than dog sera, the results in this respect being similar to the results in our study of the antihemolytic action alone of these sera.

Of interest in this connection is the relative thermostability of specific immune amboceptors and the non-specific antihemolytic and complement-inhibiting substances. For studying this relation we have selected the sera of mules immunized against glanders or suffering with that disease, also the sera of rabbits immunized with sheep cells and various bacteria and containing specific antibodies in addition to the non-specific complement-inhibiting substances. Mule sera were selected in addition to dog and rabbit sera because they behave very much as the latter in complement-fixation tests.

Heating these sera at 62 C. removed to a large degree, but not entirely, the non-specific complement-inhibiting substances with but slight depreciation of the immune amboceptors, as determined by titrating each serum in amounts ranging from 0.001 to 0.2 c.c. with constant doses of antigen; at 65 C. the non-specific reaction is still more influenced, while at 70 C. the non-specific reaction was not in evidence, altho in those sera containing large amounts of specific antibodies specific complement fixation occurred with the larger doses of serum.

TABLE 5

Complement Fixation with Normal Dog Serum (0.1 c.c.) and Normal Rabbit Serum (0.2 c.c.) Active and Heated at 56 C. for Varying Intervals of Time and with Various Lipoidal and Bacterial Antigens

Exposure, Min.	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Extract Acetone Insoluble Lipoids of Heart	Staphylococci	B. coli	Serum Control
	Serum 3 (Dog)					**Serum 9 (Dog)**					**Serum 11 (Dog)**				
Active	−	−	+++	+++	−	−	−	++	++	−	−	−	+	+	−
10	−	−	+++	+++	−	−	−	++	++	−	−	−	++	++	−
20	+	++	+++	+++	−	+	−	+++	+++	−	−	−	+++	+++	−
30	+++	+++	+++	+++	+	+++	+++	+++	+++	−	+	+	+++	+++	−
45	+++	+++	+++	+++	++	+++	++	+++	+++	−	++	+	+++	+++	−
60	+++	++	+++	+++	++	+++	++	+++	+++	−	++	+	+++	+++	−
90	++	++	+++	+++	+	+++	++	+++	+++	−	+	+	+++	+++	−
	Serum 41 (Rabbit)					**Serum 30 (Rabbit)**					**Serum − (Rabbit)**				
Active	−	−	+	+	−	−	−	−	−	−	−	−	+	+	−
10	−	−	+	+	−	−	−	−	−	−	−	−	++	++	−
20	+	+	+++	+++	−	−	−	−	−	−	+	+	+++	+++	−
30	+++	+++	+++	+++	−	−	−	−	−	−	+++	+	+++	+++	−
45	+++	+++	+++	+++	−	−	−	−	−	i	+++	+	+++	+++	−
60	+++	+++	+++	+++	−	−	−	−	−	−	+++	+	+++	+++	−
90	+	+	+++	+++	−	−	−	−	−	−	+	−	+++	+++	−

Table 6 shows these results with the serum of a glanderous mule. While the specific glanders antibody withstood the higher temperatures in sufficient degree to absorb complement, whereas the non-specific substances were in large part, or entirely, removed, it is apparent that both are affected by the same temperatures, and the greater absorption of complement in the complement-fixation tests is due to the antilytic action of serum and antigen alone in addition to specific absorption by the antigen antibody combination. Of practical importance is the observation that heating a serum at 62 to 65 C. for one-half hour largely removes the non-specific factor without total destruction of the specific antibodies.

TABLE 6

COMPARATIVE THERMOSTABILITY OF SPECIFIC GLANDERS AMBOCEPTORS AND NON-SPECIFIC ANTILYTIC SUBSTANCES

Dose c.c.	Heated at 56 C.				Heated at 65 C.				Heated at 70 C.			
	Complement-Fixation Test*		Antilytic Test		Complement-Fixation Test		Antilytic Test		Complement-Fixation Test		Antilytic Test	
	I	II	I	II	I	II	I	II	I	II	I	II
0.001	+	—	—	—	—	—	—	—	—	—	—	—
0.005	+	—	—	—	+	—	—	—	—	—	—	—
0.01	++	—	—	—	++	—	—	—	±	—	—	—
0.05	+++	++	+	—	+++	+	—	—	++	—	—	—
0.1	++++	++++	+++	++	++++	+++	++	—	++	+	—	—
0.2	++++	++++	+++	++	++++	++++	++	+	+++	+++	—	—

* The complement-fixation tests were conducted with one quarter of the antilytic dose of a glanders antigen. For the sera of glanderous animals and glanders antigen I am indebted to Dr. Fred Boerner of the State Live Stock and Sanitary Board of Pennsylvania.

THE RELATION OF COMPLEMENTOIDS AND AMBOCEPTOIDS TO NON-SPECIFIC COMPLEMENT FIXATION

As previously stated, Ehrlich and Sachs found that at 60 C., but not at 50 C., dog serum becomes antihemolytic. According to their view, the zymotoxic group is destroyed by the higher temperatures and complementoid produced, so that in hemolytic tests the complementoid would be capable of union with amboceptors without exerting lytic activity and in this manner would block the action of complement. They also observed that the haptophoric group is weakened, since complementoid exerts a weaker combining power for amboceptor than the original complement. Neisser and Friedemann[13] sought to explain the phenomenon on the basis of the formation of amboceptoids. The experiments of Noguchi, however, have shown that the antilytic action of heated serum is due to anticomplementary activity, and our own experiments support this view.

[13] Berl. klin. Wchnschr., 1902, 39, p. 677.

Six selected sera were heated at 56 C. for one-half hour. The antilytic activity of these was determined as was also their power of absorbing complement with the usual lipoidal and bacterial antigens. A portion of each serum was then treated with sheep cells which had been previously sensitized with 2 units of antisheep hemolysin and washed 3 times, and a third portion of each serum with ox cells which had been previously sensitized with 10 units of antiox hemolysin, and washed to remove unbound amboceptor. These mixtures were kept at room temperature for 2 hours to permit the union of complementoid to the sensitized corpuscles, by means of the haptophoric groups of the amboceptors. At the end of this time the corpuscles were separated by centrifugation and the supernatant fluid (diluted serum) tested in the same amounts for antilytic activity and complement-fixing power with antigens.

In 4 sera digestion with blood corpuscles was followed by partial removal of the antilytic principles, while in 2 sera a slight increase in antilytic activity was apparent; non-specific complement fixation was not influenced, so that if complementoids had been produced, either they were not attached to the hemolytic amboceptors, or, if complementoids are to be regarded as playing a rôle in the process, we must surmise not only the destruction of the zymotoxic group, but also destruction, or great weakening, of the haptophoric group so that complementoid was not attached to the blood cells.

The possible relationship of amboceptoids to the process of non-specific complement fixation was then studied experimentally. The majority of dog sera contain appreciable quantities of natural antisheep hemolysin. These amboceptors were removed from 6 selected sera by means of saturation with washed sheep cells and the sera tested for their antilytic and complement-fixing powers. Three of these extracted sera, when tested unheated after digestion with blood cells, were found to have yielded stronger complement-fixation; after heating the sera at 56 C. for 30 minutes their antilytic and complement-fixing powers were practically equal to those shown by portions of each serum heated and tested without previous removal of hemolytic amboceptors.

Portions of each serum were then heated at 56 C. for one-half hour and mixed with fresh, washed sheep and ox cells and placed in the refrigerator for 18 hours to permit the union of amboceptoid (hemolytic) to the cells by means of their cytophilic arms. At the end

of this time the mixtures were centrifugated and the sera separated. These sera were then tested for their antilytic and complement-fixing powers. In 3 sera the antilytic activity was slightly increased, and in 3 other sera there was either removal or weakening of the antilytic and complement-fixing power. These results were similar to those shown in Tables 7 and 8.

Both these experiments were repeated with emulsions of Staphylococcus, B. coli, and B. typhosus, instead of emulsions of erythrocytes, with the object of dealing with any amboceptors for these that might

TABLE 7

ANTILYTIC PRINCIPLES OF HEATED DOG SERUM SLIGHTLY REMOVED BY SENSITIZED BLOOD CELLS

Amount of Serum	Dog 42 (56 C.)		Dog 80 (56 C.)		Dog 96 (56 C.)		Dog 142 (56 C.)	
	Before Digestion	After Digestion	Before Digestion	After Digestion	Before Digestion	After Digestion	Before Digestion	After Digestion
0.005	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.01	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.05	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Trace	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.1	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis	Complete hemolysis
0 15	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Moderate hemolysis (slightly antilytic)	Moderate hemolysis (slightly antilytic)	Complete hemolysis	Complete hemolysis
0.2	Moderate hemolysis (slightly antilytic)	Marked hemolysis	Complete hemolysis	Moderate hemolysis (slightly antilytic)	None	None	Moderate hemolysis (slightly antilytic)	Marked hemolysis

be present. The results were identical with those observed with the corpuscles, and it is difficult to account for the antilytic and complement-fixing powers of these sera on the basis of amboceptoid formation.

REGARDING ABSORPTION OF THE ANTILYTIC AND COMPLEMENT-FIXING SUBSTANCES OF DOG SERUM BY BLOOD CORPUSCLES

According to Noguchi, corpuscles of various animals can deprive heated sera of their antilytic principles, the corpuscles becoming more resistant to serum hemolysis. As stated above, when we digested heated sera with washed sheep or ox cells, the antilytic activity of the

TABLE 8

NON-SPECIFIC COMPLEMENT-ABSORBING SUBSTANCES OF HEATED DOG SERUM NOT REMOVED BY SENSITIZED BLOOD CELLS (SERUM HEATED AT 56 C. DOSE 0.2 c.c.)

Dog	Before Digestion							After Digestion						
	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	B. coli	Staphylococci	B. typhosus	Serum Control	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Extract Acetone Insoluble Lipoids of Heart	B. coli	Staphylococci	B. typhosus	Serum Control
42.............	+	−	−	++	++	++	+	+	−	−	+++	++	++	±
80.............	++	+	+	+++	+++	+++	−	++	+	+	++++	++++	++++	+
96.............	+++	++	++	++++	++++	++++	++	+++	++	++	++++	++++	++++	++
142.............	+++	++	++	++++	++++	++++	+	+++	++	++	++++	++++	++++	+

sera was increased in some instances and decreased in others. The complement-fixing powers of the sera with the various antigens employed were not influenced. But when the sheep and ox cells used in treating the heated sera were removed from the sera after 18 hours in the refrigerator, washed 3 times, and re-suspended in normal salt solution in a 2.5% suspension and compared with a similar suspension of untreated cells, preserved in the refrigerator for the same length of time, no appreciable differences in the susceptibility of the cells to lysis by the same doses of amboceptors and complement could be detected. In Table 9 are shown 4 titrations of sheep cells used in digesting the 4 heated dog sera shown in Tables 7 and 8.

TABLE 9

Sheep Corpuscles do not Become More Resistant to Serum Hemolysis as a Result of Digestion with Heated Dog Serum (Antisheep Hemolysin in Dose of 0.005 c.c. with 0.05 c.c. Mixed Guinea-pig Serum Complement)

Amount of Cells, c.c.	Dog 42		Dog 80		Dog 96		Dog 142	
	Cells Before Digestion	Cells After Digestion	Cells Before Digestion	Cells After Digestion	Cells Before Digestion	Cells After Digestion	Cells Before Digestion	Cells After Digestion
0.05	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.1	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.3	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.5	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.8	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
1.0	Moderate hemolysis (slightly hemolytic)	Moderate hemolysis (slightly hemolytic)	Moderate hemolysis (slightly hemolytic)	Moderate hemolysis (slightly hemolytic)	Moderate hemolysis (slightly hemolytic)	Moderate hemolysis (slightly hemolytic)	Moderate hemolysis (slightly hemolytic)	Moderate hemolysis (slightly hemolytic)

According to our experiments, therefore, blood corpuscles act indifferently in the absorption of the antilytic and complement-fixing principles of dog serum; frequently the reverse was observed, the antilytic activity of the serum being increased. Similar results were observed in the following set of experiments.

RELATION OF BACTERIOLYTIC AMBOCEPTORS TO NON-SPECIFIC COMPLEMENT FIXATION BY DOG AND RABBIT SERA

Because of the fact that a large percentage of normal dog and rabbit sera absorb complement to a well-marked degree in the presence

of various bacterial antigens, as pointed out in our previous papers, it was necessary to learn whether bacteriolytic amboceptors were present, altho the reactions gave indications of being non-specific.

We have not tested the bacteriolytic power of serum, but have studied the question by means of absorption tests. Six dog, and 9 rabbit sera were heated at 56 C. for one-half hour and used in complement-fixation tests in dose of 0.2 c.c. with lipoidal and 3 bacterial antigens (Staphylococcus, B. coli, and B. typhosus). Two cubic centimeters of each serum were then absorbed for 18 hours with 0.2 c.c. of a thick, washed emulsion of each of the three bacterial cultures. At the end of this time the mixtures were subjected to prolonged centrifugation at high speed and the clear sera separated from the sediment of bacteria and tested in the same manner with the same antigens.

As shown in Table 10, absorption with these bacteria tends to remove a portion of the principles in the sera responsible for the fixation of complement with these antigens. On the other hand, absorption appeared to increase the antilytic power of some sera, as well as the degree of complement fixation with the antigens, acting in both these respects in a manner similar to that of absorption with blood corpuscles. Likewise, the absence of specificity is evidence against the rôle of specific amboceptors or amboceptoids in the process, as digestion of serum with staphylococci absorbs a portion of the principles reacting with such antigens as alcoholic extract of heart, B. coli, and B. typhosus, as well as with Staphylococcus; similar results were observed after digestion with B. coli and B. typhosus . It would appear therefore, that the antilytic and complement-fixing principles are nonspecific and capable of partial absorption, not only by blood corpuscles, but by bacterial cells as well.

THE RELATION OF PARASITES TO NON-SPECIFIC COMPLEMENT FIXATION BY NORMAL DOG AND RABBIT SERA

Coccidiosis in rabbits has been regarded by a few investigators as a probable factor in the phenomenon of non-specific complement fixation. Among the animals in our previous study[14] and likewise among those of the present series, coccidiosis was relatively frequent, but we have not been able to trace any relation between this disease and the property in the sera of non-specific complement fixation—animals

[14] Kolmer and Casselman: Jour. Med. Research, 1913, 28, p. 369.

TABLE 10

Influence of Absorption by Bacteria on the Antilytic and Complement-fixing Powders of Dog and Rabbit Sera

Serum 56 C.	Before Absorption					After Absorption with Cocci					After Absorption with B. coli					After Absorption with B. typhosus				
	Cholesterinized Alcoholic Extract of Heart	Staphylococci	B. coli	B. typhosus	Serum Control	Cholesterinized Alcoholic Extract of Heart	Staphylococci	B. coli	B. typhosus	Serum Control	Cholesterinized Alcoholic Extract of Heart	Staphylococci	B. coli	B. typhosus	Serum Control	Cholesterinized Alcoholic Extract of Heart	Staphylococci	B. coli	B. typhosus	Serum Control
Dog 1...	++	++++	++	+++	+	++	++++	++	++++	++	++	++++	++	++++	+	++	++++	++	++++	+
Dog 2...	+	++	++	++	–	+	+++	+++	+++	–	+	++	+++	+++	–	+	++	+++	+++	–
Dog 3...	+++++	+++++	+++++	+++++	++	++	+++++	++	++	++	++	+++++	++	++	++	++	+++++	++	++	++
Dog 4...	+++++	+++++	+++++	+++++	+	+	+++++	++	++	+	+	+++++	++	++	+	+	+++++	++	++	+
Dog 5...	+++++	+++++	+++++	+++++	++	+	++	+	+	+	+	++	+	+	+	+	++	+	+	+
Dog 6...	+++++	+++++	+++++	+++++	++	–	+++	–	–	–	–	+++	–	–	–	–	+++	–	–	–
Rabbit 1	–	+++++	+	++	–	++	+++	++	++	++	++	+++	++	++	++	++	+++	++	++	+
Rabbit 2	±	+++++	+	+	–	+	+++	++	++	+	+	+++	++	++	+	+	+++	++	++	+
Rabbit 3	–	++	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	+	–	–
Rabbit 4	+++++	+++++	+++++	+++++	–	–	++	–	–	–	–	++	++	–	–	–	++	–	–	–
Rabbit 5	–	+++	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	++	–	–
Rabbit 6	–	+++	–	–	–	+	+	–	–	–	–	–	+	–	–	–	–	+	–	–

with advanced lesions have reacted negatively, and young rabbits with no evidences of the infection have reacted positively.

Many of the dogs used in these studies have been examined post mortem in the hope of finding some lesion or some parasitic infection that might explain the positive reaction. Parasites, as for example Cysticercus pisiformis and Coccidium cuniculi, have frequently been found, but these appear to bear no relation to the occurrence of a positive reaction.

We have paid particular attention to the probable relation of intestinal parasites in dogs to non-specific complement fixation and to the question of specific complement fixation in intestinal parasitism (the results of these studies are given elsewhere), but we do not believe that these parasites are directly concerned in producing the principles in the serum responsible for the antilytic properties of dog serum and its tendency to absorb complement in a non-specific manner with lipoidal tissue extracts and bacterial antigens.

THE INFLUENCE OF SALVARSAN UPON NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT SERUM *

Emanuel[15] claims that the administration of salvarsan tends to remove the property in rabbit serum of absorbing complement in a non-specific manner. Epstein and Pribram[16] have made a similar claim for mercury.

In the past year, during chemotherapeutic researches, we have used rabbits for determining the lethal dose of salvarsan, and have performed complement-fixation tests with their sera before, and at varying intervals after, the intravenous administration of 70 milligrams per kilogram of body weight. This dose represents 4.2 grams for a man weighing 60 kilos, and is the maximal dose of the usual preparation of salvarsan that may be administered to rabbits with safety. The results are shown in Table 11. Occasionally a slight weakening in the reactions with inactivated serum was noted, but, as a general rule, the administration of a single large dose of salvarsan was without effect upon the property of normal rabbit serum for absorbing complement in a non-specific manner.

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15 Berl. klin. Wchnschr., 1911, 48, p. 2335.

16 Ztschr. f. exper. Path. u. Therap., 1909, 7, p. 549.

The Influence of Salvarsan upon Non-specific Complement Fixation with Normal Rabbit Serum

No.	Salvarsan (Intravenous Injection)	Active Serum (0.2 c.c.)					Inactivated Serum (0.2 c.c.)				
		Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Staphylococci	B. coli	Serum Control	Cholesterinized Alcoholic Extract of Heart	Alcoholic Extract of Syphilitic Liver	Staphylococci	B. coli	Serum Control
44	Before salvarsan	—	—	+++	++	—			+++	+++	—
44	36 hr. after 70 mg. per kilo	—	—	++++	++++	—			+++	+++	—
44	14 days later	—	—	+++	+	—			+++	+++	—
44	21 days later	—	—	+++	++++	—			+++	+++	—
33	Before salvarsan	++++	++++	++++	++++	—	++	++	+++	+++	—
33	4 days after 70 mg. per kilo	—	—	+	—	—	+++	+++	+++	+++	—
33	12 days later	—	—	—	—	—	+++	+++	+++	+++	—
33	28 days later	—	—	—	—	—	+++	+++	+++	+++	—
29	Before salvarsan	—	—	—	—	—	+++	+++	+++	+++	—
29	48 hr. after 100 mg. per kilo	—	—	—	—	—	+	+	+++	+++	—
29	28 days later	—	—	—	—	—	+++	+++	+++	+++	—
29	40 days later	—	—	—	—	—	+++	+++	+++	+++	—
29	45 days later	—	—	—	—	—	++	++	+++	+++	—
37	Before salvarsan	—	—	—	—	—	+++	+++	+++	+++	—
37	Immediately after 70 mg. per kilo	—	—	—	—	—	+	+	+++	+++	—
37	24 hr. later	—	—	—	—	—	+++	+++	+++	+++	—
60	Before salvarsan	—	—	—	—	—	++	++	+++	+++	—
60	24 hr. after 70 mg. per kilo	—	—	—	—	—	+++	+++	+++	+++	—
60	5 days later	—	—	—	—	—	++	++	+++	+++	—
62	Before salvarsan	—	—	—	—	—	+++	+++	+++	+++	—
62	24 hr. after 70 mg. per kilo	—	—	—	—	—	+++	+++	+++	+++	—
62	5 days later	—	—	—	—	—	+++	+++	+++	+++	—
62	49 days later	—	—	—	—	—	+++	+++	+++	+++	—
27	Before salvarsan	—	—	—	—	—	+++	+++	+++	+++	—
27	Immediately after 70 mg. per kilo	—	—	—	—	—	++	++	+++	++	—
28	Before salvarsan	—	—	—	—	—	+++	+++	+++	+++	—
28	24 hr. after 70 mg. per kilo	—	—	—	—	—	++	++	+++	+++	—
28	96 hr. later	—	—	—	—	—	++	+	+++	+	—

Mercury likewise appears to have no influence on this property of rabbit sera. We have administered daily intramuscular injections of soluble salts over a period of several weeks without definite influence on the serum reactions.

QUANTITATIVE FACTORS IN NON-SPECIFIC COMPLEMENT FIXATION WITH NORMAL RABBIT AND DOG SERA

As in all complement-fixation tests, quantitative factors have a direct and considerable influence on the results. In this and previous papers we have emphasized the importance of quantitative factors in non-specific complement fixation with rabbit and dog sera. As is well known, an excess of antigen, a decrease of complement, and a serum containing thermostabil anticomplementary bodies are factors which may produce false positive reactions with any serum; conversely, an excess of complement or hemolysin tends to mask absorption or fixation of complement, yielding false negative reactions.

In all our work the lipoidal extracts were used in doses equal to 6 to 12 times less than their anticomplementary doses, and the bacterial emulsions in one-quarter of their anticomplementary doses. The use of larger doses tended to increase the percentage of non-specific reactions, and smaller doses yielded fewer reactions.

As the serum complements of guinea-pigs vary in their adaptability for fixation, the mixed sera of at least 2 pigs were generally used in dose of 0.05 c.c. (1 c.c. of a 1:20 dilution). This dose of complement has been found reliable and was used as a fixed unit in titrating the hemolysin against a fixed dose of corpuscle suspension (1 c.c. of a 2.5% suspension of washed sheep cells). The hemolysin was used in an amount equal to double the unit, being titrated each day with the complement serum and corpuscles. The use of a smaller dose of hemolysin increases the tendency for non-specific reactions, and the so-called "single unit" system should never be used in conducting complement-fixation tests with rabbit and dog sera.

With the technic employed by us the amount of complement absorbed was rather variable with the sera of different animals. When Kolmer's method for determining the degree of complement fixation according to the number of units of hemolytic complement absorbed, was used, of the lipoidal extracts most absorption occurred with the alcoholic extract of heart re-enforced with cholesterin, and with bacterial antigens more complement was usually absorbed than with the lipoidal extracts. As a general rule, dog serum absorbed more com-

plement than did rabbit serum, and in all instances heated serum more than unheated. Even with sera yielding strong reactions according to the usual technic, the amount of complement absorbed according to the quantitative method was relatively small and generally much less than that absorbed by the serum of a syphilitic person with the ordinary lipoidal extracts.

A large proportion of fresh rabbit sera contain natural or native antisheep hemolysin. Kolmer and Williams[17] found that 36% of rabbit sera in doses of 0.2 c.c. contain sufficient antisheep hemolysin to cause complete lysis of 1 c.c. of a 2.5% suspension of cells with 0.05 c.c. complement, the same quantities used in the complement-fixation tests of this work. Likewise, a large proportion of normal dog sera contain antisheep hemolysin, and usually 0.2 c.c. of fresh dog serum contains sufficient hemolysin and complement to cause complete lysis of 1 c.c. of a 2.5% suspension of sheep cells.

The presence of these natural amboceptors in addition to complement in fresh unheated serum probably explains in part the smaller percentage of non-specific reactions when active serum is used, slight degrees of complement absorption being masked.

With sera, however, in which the hemolytic complement and anti-sheep hemolysin are titrated and known, the well-defined absorption or fixation of complement that follows with sera heated at 55 C. for one-half hour, as compared with unheated sera, cannot be entirely explained on the basis that the native complement has been inactivated and native hemolysin possibly weakened; on the contrary, some chemical change has probably occurred in the nature of an alteration of the colloidal conditions, so that in a mixture of serum, complement, and antigen, complement is absorbed in a non-specific manner.

It is to be emphasized that in conducting complement-fixation tests with dog and rabbit sera, the antigen should be carefully titrated, and not used in an amount over one-quarter of its anticomplementary dose; if the serum is used after inactivation, the dose of complement should be sufficiently large, and the immune hemolysin should be used in an amount equal to at least double its hemolytic unit.

DISCUSSION OF THE NATURE OF NON-SPECIFIC COMPLEMENT FIXATION BY NORMAL RABBIT AND DOG SERA

While it is not possible to make definite statements, on the basis of experimental evidence, regarding the nature of the substances in serum

[17] Jour. of Med. Research, 1913, 28, p. 369.

responsible for non-specific complement fixation, it is highly probable that these substances are similar to, or identical with, the antilytic substances. The slight differences may be only apparent, and just as a human serum may show complete hemolysis in a serum control-tube of a complement-fixation test and yet be slightly anticomplementary, yielding well-marked non-specific inhibition of hemolysis in the presence of the antigen, so in like manner the complement-absorbing properties of dog and rabbit sera in complement-fixation tests stand out more prominently than the purely antilytic powers.

In our opinion, the phenomenon is entirely non-specific, the complement fixation being due to the antilytic substances plus the antilytic properties of even minute amounts of the extracts and emulsions used as antigens.

Objections may be raised therefore, against calling this process non-specific complement fixation. We have purposely adhered to this terminology because the phenomenon may be detected only in complement-fixation tests and it is probable that the antilytic substances may, with the antigen, truly absorb, or fix, complement. While the antilytic substances are now believed to be anticomplementary, the exact influence upon complements is not known.

In complement-fixation tests with dog and rabbit sera the antilytic tendency of the serum may not be in evidence because of ready and complete hemolysis of the serum control-tubes, and yet some degree of inhibition of hemolysis may result in those tubes containing antigen. We have sought to emphasize the importance of these non-specific reactions because of their bearing upon practical and experimental work with these sera, in addition to the light that may be thrown on the mechanism of complement fixation in general, and upon the Wassermann reaction in particular.

CONCLUSIONS

1. Non-specific complement fixation by normal rabbit and dog sera is probably due primarily to thermolabil and thermostabil antilytic (anticomplementary) substances in the sera.

2. While fresh and active rabbit and dog sera may yield non-specific complement-fixation, the tendency is greatly increased as a result of heating the sera. At 56 C. the changes may occur in 20 minutes or even less; at 62 C. for 30 minutes the tendency for non-specific reactions is much decreased, and is entirely removed by heating

serum at 70 C. for 30 minutes. Changes may occur after exposure at 45 C. for 30 minutes, but the optimal temperature is between 55 and 60 C.

3. In complement-fixation tests for specific antibodies with inactivated rabbit, dog, and mule sera, it is advisable to heat the sera at 62 C. for one-half hour and to use at least 2 units of complement or hemolysin and no more than one-quarter of the anticomplementary unit of antigen after it has been carefully titrated.

4. Complementoids and amboceptoids probably bear no relation to the process of non-specific complement fixation by rabbit and dog sera.

5. The blood corpuscles of various animals and various bacteria may absorb a portion of the antilytic substances from rabbit and dog sera, but they have much less influence on the complement-fixation reactions. Digestion of fresh sera with corpuscles and bacteria not infrequently increases the anticomplementary properties of the sera.

6. Bacteriolytic amboceptors are not responsible for non-specific complement fixation by normal rabbit and dog sera.

7. Parasitic infestations of rabbits and dogs bear no relation to the antilytic and complement-fixing properties of the sera.

8. Single, large doses of salvarsan are without definite influence on the reactions with rabbit serum.

9. Quantitative factors in the hemolytic system and antigen are of considerable importance in relation to these non-specific reactions.

10. If time permits, preliminary complement-fixation tests should be performed with the sera of rabbits or dogs before immunization or inoculation is begun, and only those animals selected the sera of which react negatively with the antigen used.

COMPLEMENT FIXATION IN INTESTINAL PARASITISM OF DOGS *

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Infestation of the intestines with the commoner varieties of parasites is generally regarded as exercising no particular harm on the host, except to produce such general disturbances as digestive derangements or anemia, the latter being ascribed to the loss of blood through blood-sucking parasites and to the absorption of hemotoxic substances. Examples are not wanting, however, both among men, and among animals—notably the dog and the horse—of blood changes and many symptoms of disease which may be ascribed to the production by the parasites of toxic substances, capable of being absorbed and exercising harmful effects.

The object of our study was to determine by means of a complement-fixation technic whether absorption of foreign substances with the production of antibodies occurred in dogs infested with the common varieties of intestinal parasites. That production of antibodies may result from superficial infections is shown by the immunologic studies in parasitic diseases of the skin by Kolmer and Strickler,[1] in which it was found that in ring-worm of the scalp and favus, by means of a complement-fixation technic specific antibodies might be detected in the blood serum in a large majority of diseased individuals. In ring-worm particularly, the fungus seldom penetrates to the deeper layers of the epidermis and rarely to the corium; hence, it may be assumed that soluble toxic substances are produced by the fungus, which being absorbed cause the production of antibodies. The encouraging results of this work induced us to undertake a similar study of intestinal parasitism, dogs being selected on account of the frequency with which they are infested and the means offered for controlling the results by frequent examinations of the feces and by autopsies.

[1] Jour. Am. Med. Assn., 1915, 64, p. 800.

Historical Review

Several investigators have employed a complement-fixation technic in the diagnosis of echinococcus disease of the liver. Ghedini[2] found this test of value, and he also reports specific reactions with the sera of persons infested with Ankylostoma duodenales and Ascaris lumbricoides.[3] Weinberg,[4] Jiani,[5] Israel,[6] and Henius[7] report favorable and specific reactions in echinococcus disease with aqueous or alcoholic extracts of cyst fluid or both. Kurt Meyer[8] found these reactions non-specific, in that the serum of a person infested with echinococcus showed complement-absorption with antigens of Tenia solium and T. saginata, and vice versa. Branes[9] found that alcoholic extracts of echinococcus cyst fluid showed complement-absorption with the syphilis antibody as well as with that of echinococcus disease, and this observation has been generally confirmed. Thomsen and Magnusson[10] in a study of 12 cases of echinococcus disease found that the sera of 10 reacted positively; the sera of 55 control cases (32 of which reacted positively to the Wassermann reaction) all were negative except one. These authors also report favorably on the specificity of the reaction; the sera of 10 persons infected with Tenia saginata, of 2 with T. solium, and of 1 with Bothriocephalus latus, all were negative with echinococcus antigen.

In so far as echinococcus disease of the liver is concerned, a review of the literature shows a general consensus of opinion that antibodies are present in the sera of the majority of diseased persons and animals and that these may be detected by means of a complement-fixation test. There is, however, a division of opinion in regard to the specificity of the reaction and its practical value in diagnosis.

Much less work has been reported on complement fixation with sera of persons infested with such parasites as Tenia saginata, Ascaris lumbricoides, etc. Of interest in this connection, as showing the probability of antibody-formation in infestations with these parasites and in protozoal infections in general, are the reports of Rubinstein and Julien,[11] Manoileff,[12] Gozony,[13] and Hindle and Gozony,[14] who found ferments in the sera of individuals, as determined by the methods of Abderhalden, using as substrates preparations of T. solium and T. saginata, Ascaris lumbricoides, etc.

Materials and Methods of Study

Antigens.—The following extracts of parasites, secured from dogs at autopsies, were prepared:

1. Salt-solution extract of Tenia serrata.
2. Alcoholic extract of T. serrata.

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[2] Gaz. degli Ospedali e delle Cliniche, 1906, 27, p. 1616; 1907, 28, p. 53.

[3] Ibid., 1907, 28, p. 476.

[4] Ann. de l'Inst. Pasteur, 1909, 23, p. 472 (in which references are given to his previous work in this field).

[5] Wien. klin. Wchnschr., 1909, 22, p. 1439 (in which the author gives a good bibliography of earlier literature).

[6] Ztschr. f. Hyg. u. Infektionshrankh., 1910, 66, p. 487.

[7] Deutsch. med. Wchnschr., 1911, 37, p. 1212.

[8] Berl. klin. Wchnschr., 1910, 47, p. 1316.

[9] München. med. Wchnschr., 1911, 58, p. 1073.

[10] Berl. klin. Wchnschr., 1912, 49, p. 1183.

[11] Compt. rend. Soc. de biol., 1913, 75, p. 180. Abstract by Graltz, Ztschr. f. Immunitätsf. R., 1913, 8, p. 63.

[12] Wien. klin. Wchnschr., 1914, 27, p. 269.

[13] Centralbl. f. Bakteriol., I, O., 1914, 73, p. 345.

[14] Parasitology, 1914, 7, p. 228.

3. Salt-solution extract of Dipylidium caninum.
4. Alcoholic extract of Dipylidium caninum.
5. Salt-solution extract of Ascaris canis.
6. Alcoholic extract of Ascaris canis.
7. Salt-solution extract of Trichocephalus dispar (whip worm).
8. Salt-solution extract of Strongylus gigas.
9. Alcoholic extract of T. saginata.

Antigen 9 was prepared from a parasite from a human host and is included in this series for the purpose of studying the specificity of the reactions with parasites from dogs.

Strongylus gigas is found in the pelvis of the kidney, and while, accordingly, it is not an intestinal parasite or a common type of infestation, we thought it would be of interest to include this antigen in our series.

These parasites, representing the commoner varieties to be found in dogs in this district, sufficed for the purposes of this work; namely, to ascertain whether their presence in an animal was followed by the production of antibodies that could be detected in the serum by means of a complement-fixation test.

In the preparation of each salt-solution antigen the fresh parasites were washed several times, and 4 grams thoroughly ground and macerated with sand and powdered glass and then suspended in 100 c.c. of sterile salt solution containing 0.5% phenol. This mixture was shaken mechanically for 24 hours, incubated at 37 C. for several days, and then filtered and stored in the refrigerator.

The alcoholic extracts were prepared in the same manner with the use of absolute ethyl alcohol, except that extraction in the incubator was continued for a longer period of time.

Antigens prepared in this manner contained a portion of the protein and other constituents of the parasite, as well as endotoxic substances, and generally proved satisfactory in the titrations and complement-fixation tests.

All antigens were diluted with salt solution and titrated at frequent intervals, usually just before the complement-fixation tests, and used in amounts corresponding to one-quarter of their anticomplementary doses. The salt-solution extracts were found to vary in their anticomplementary units to such an extent as to require titration before every experiment; the alcoholic extracts were more stable.

Table 1 shows the method of titration and the doses employed, and is representative of these titrations.

Hemolytic System.—The antisheep hemolytic system was employed. Complement was furnished by the mixed sera of 2 or more guinea-pigs, and was used in dose of 0.05 c.c. (1 c.c. of 1:20 dilution). Antisheep hemolysin was titrated each time against this constant dose of complement and 1 c.c. of a 2.5% suspension of sheep cells, and was used in the antigen titrations and in complement-fixation tests in amounts equal to 2 hemolytic units.

In the anticomplementary titrations of the antigens increasing amounts of antigen (Table 1) were incubated with the complement for 1 hour at 37 C.; then 2 units of hemolysin and 1 c.c. of the corpuscle suspension were added, the whole mixed, re-incubated for 1 hour, and the results read.

In the complement-fixation tests fresh or inactivated sera in varying doses were incubated with the various antigens and complement for 1 hour; then 2 units of hemolysin and 1 c.c. of corpuscle suspension were added; after mixing and re-incubation for 1 hour or over, according to the hemolysis of the con-

trols, the results were either read at once, or the tubes were placed in a refrigerator and the reading made the following morning. As usual every serum and antigen and hemolytic system was controlled in each set of titrations and reactions.

Sera.—In all, the sera of 172 dogs were examined. As will be pointed out later in this paper, our greatest difficulty in this work was the tendency of dog serum to yield non-specific complement fixation. For this reason the sera were generally used in an active state or after inactivation at 62 C., and in various doses, ranging from 0.05 to 0.4 c.c. — usually 0.1 c.c. Blood was collected from each dog from the external jugular vein by means of sterile needles in sterile test tubes, and in those tests in which active serum was used, the reactions were conducted within 24 hours after the collection.

TABLE 1

Anticomplementary Titrations of Antigens

Amount c.c.	Salt Solution Extract T. serrata	Alcoholic Extract T. serrata	Salt Solution Extract of Dipylidium caninum	Alcoholic Extract Dipylidium caninum	Salt Solution Extract Ascaris canis	Alcoholic Extract Ascaris canis	Salt Solution Extract Trichocephalus dispar	Salt Solution Extract Strongylus gigas	Alcoholic Extract T. saginata
	1:10	1:5	1:20	1:20	1:5	1:20	1:4	1:10	1:20
0.2	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.4	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.6	Marked hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Marked hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.8	Slight hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
1.0	No hemolysis	Complete hemolysis	Complete hemolysis	Marked hemolysis	Complete hemolysis	No hemolysis	Marked hemolysis	Complete hemolysis	Complete hemolysis
2.0	No hemolysis	Slight hemolysis	Slight hemolysis	Slight hemolysis	Marked hemolysis	No hemolysis	Slight hemolysis	Marked hemolysis	Marked hemolysis
Dose	0.1	0.4	0.4	0.25	0.5	0.1	0.25	0.5	0.5

Method of Study.—Each serum with few exceptions was tested with all the antigens. The results were checked up by examinations of the feces of the animals for ova and parasites and by autopsies. In our earlier work a few days or a week or more would elapse between serum tests and feces examinations, but because parasitic conditions may vary from time to time, feces were collected on the day of the collection of blood, or on the day following. Each specimen of feces was examined macroscopically and microscopically (in the latter case by the direct smear and centrifuge methods) for parasites and ova.

We wish to express our appreciation of the aid given us by Dr. Allen J. Smith and Dr. Damaso Rivas in the identification of ova and parasites in numerous instances.

DIFFICULTIES ENCOUNTERED IN THE WORK

In this study we were confronted with two main difficulties. The first was the tendency of dog serum to yield non-specific complement-fixation. We have particularly studied this phase of the subject, the results being given in a separate communication.[15] To overcome this non-specific factor, we have carefully titrated our antigens and used them in doses equal to one-quarter or less of their anticomplementary units; the hemolysin was used in amounts equal to, or double the hemolytic unit, and the sera were used in a fresh, active condition and again after inactivation, in doses varying from 0.05 to 0.4 c.c. Sera that proved anticomplementary in the complement-fixation tests were excluded; best results were secured with fresh and active sera in dose of 0.1 c.c., in which native dog complement and antisheep hemolysin aided in overcoming the antilytic and non-specific complement-fixation tendencies of the serum, or, after heating the sera at 62 C., instead of 56 C., for 30 minutes.

The second difficulty was our inability to ascertain how long a dog had been infested with a particular parasite, together with the difficulty of excluding infestations on the basis of single, or even multiple, negative examinations of the feces. As will be shown later, we have observed positive complement-fixation with various antigens among 28 dogs (Table 5) in the feces of which ova were not found. It is an open question whether one or two negative feces examinations should exclude intestinal parasitism, or, whether antibodies could not persist in the body fluids for some time after the expulsion of a parasite or parasites.

RESULTS

Serum tests and feces examinations were made of 110 dogs. The results of the feces examinations may be summarized as follows:

25 dogs, or 23%, showed the presence of the ova of Ascaris canis.

16 dogs, or 14.5%, showed the presence of ova Ascaris canis and Trichocephalus dispar (whip-worm).

7 dogs, or 6%, showed the presence of ova of Tenia serrata.

4 dogs, or 3.6%, showed the presence of the ova of Dipylidium caninum.

15 Kolmer, Trist, and Heist: Jour. Infect. Dis., 1916, 18, p. 27.

22 dogs, or 20%, showed the presence of the ova of Trichocephalus dispar.

7 dogs, or 6%, showed the presence of the ova of Trichocephalus dispar and Uncinaria canina (Ankylostoma caninum).

29 dogs, or 26%, showed no ova or parasites in the feces.

Complement-fixation tests were made with 172 sera, including the 110 dogs which had had feces examinations. The sera of 62 additional dogs were examined, but the animals were removed before thorough examination of the feces could be made; accordingly, this group has not been included in an analysis of the results for this report.

The results of the complement-fixation tests did not tally with the results of the feces examinations. The important difference does not lie so much in the fact that dogs showed the ova of a certain parasite in the feces while their sera were negative with an antigen of that parasite in a complement-fixation test, as in the observation that the sera of many dogs reacted with the antigen of parasites the ova of which could not be found in the feces. As previously stated, we had no method of ascertaining whether or not these animals had been infested at an earlier period and the antibodies were still present in the blood, or whether the parasites were present in the intestinal tract with so few ova in the feces as to escape detection. The results, therefore, may be summarized in two ways: (1) Results of complement-fixation tests with the antigen or antigens corresponding to the ova found in the feces compared with the results of feces examinations; and (2) results in complement-fixation tests with the various antigens regardless of the results of feces examinations.

Tables 2 to 4, inclusive, show the results of complement-fixation tests with the sera of dogs the feces of which had been examined and classified according to the ova found; Table 5 shows the results of complement-fixation tests with the sera of dogs the feces examinations of which had yielded negative results.

The results of complement-fixation tests in Ascaris canis infestations, shown in Table 2, may be summarized as follows:

1. Of 36 dogs, the sera of 10, or about 30%, reacted positively with a salt-solution extract of Ascaris canis.

2. The sera of but 2 of these dogs, or about 5.5%, reacted positively with an alcoholic extract of Ascaris canis.

3. With an alcoholic extract of Ascaris lumbricoides positive reactions were observed with the sera of 11 dogs, or about 30%, and 5 of

TABLE 2

Results of Complement-fixation Tests in Infestations with Ascaris Canis and Trichocephalus Dispar

Dog	Serum	Dose Serum, c.c.	Salt-Solution Extract T. Serrata	Alcoholic Extract T. Serrata	Salt-Solution Extract Dipylidium Caninum	Alcoholic Extract Dipylidium Caninum	Salt-Solution Extract Ascaris Canis	Alcoholic Extract Ascaris Canis	Salt-Solution Extract Trichocephalus Dispar	Salt-Solution Extract T. Strongylus Gigas	Alcoholic Extract T. Saginata	Serum Control
					Ova of Ascaris Canis in Feces							
223A	Active	0.1	—	+	—	—	−−	—	—	—	—	—
227A	Active	0.1	+++	++++	—	—	+++	—	++	+++	—	—
238A	Active	0.1	—	++++	—	—	+++	—	—	—	—	—
240A	Active	0.1	—	+	—	—	—	—	—	—	—	—
241A	Active	0.1	—	++++	—	—	—	—	—	—	—	—
245A	Active	0.1	—	+++	—	—	++	—	—	—	—	—
334	Active	0.1	—	++++	—	—	—	—	—	—	—	—
310	Active	0.1	—	—	—	—	—	—	—	—	—	—
321	Active	0.1	—	—	—	—	—	—	—	—	—	—
195A	Active	0.1	—	—	—	—	—	—	—	—	—	—
197A	Active	0.1	—	++++	—	—	—	—	—	—	—	—
201A	Active	0.1	—	—	—	—	—	—	—	—	—	—
204A	Active	0.1	—	++++	—	++	++++	+	—	+	—	—
207A	Active	0.1	—	++++	—	—	—	—	—	—	—	—
209A	Active	0.1	—	—	—	—	—	—	—	—	—	—
215A	Active	0.1	—	++++	—	—	++++	—	—	—	—	—
217A	Active	0.1	—	++++	—	+	++	—	—	—	—	—
251A	Active	0.1	—	—	—	—	—	—	—	—	—	—
449	Active	0.2	—	—	—	—	—	—	—	—	—	—
456	Active	0.2	—	—	—	—	—	—	—	—	—	—
457	Active	0.2	—	—	—	—	—	—	—	—	—	—
477	Active	0.2	—	—	—	—	—	—	—	—	—	—
385	Inactive	0.2	++++	++++	—	—	—	—	—	—	—	—
434	Inactive	0.2	—	—	—	—	—	—	0	—	—	—
					Ova of Ascaris Canis and Trichocephalus Dispar in Feces							
228A	Active	0.1	+	++++	−−	—	++++	+++	—	+++	—	—
230A	Active	0.1	—	+++	—	—	++	—	—	—	—	—
239A	Active	0.1	—	—	—	—	—	—	—	—	—	—
252A	Active	0.1	—	++++	—	—	—	—	—	—	—	—
307A	Active	0.1	—	—	—	—	—	—	—	—	—	—
198A	Active	0.1	—	—	—	—	—	—	0	—	—	—
191A	Active	0.1	—	++++	—	—	—	—	—	—	—	—
199A	Active	0.1	—	—	—	—	+	—	0	—	—	—
202A	Active	0.1	—	++++	—	—	—	—	0	—	—	—
407	Active	...	—	—	—	—	—	—	—	—	—	—
475	Active	0.1	—	—	—	—	±	—	—	—	—	—
427	Inactive	0.1	++++	++++	—	—	—	—	0	0	—	—

Key to Tables

++++ = 100% inhibition of hemolysis (strongly positive)
+++ = 75% inhibition of hemolysis (moderately positive).
++ = 50% inhibition of hemolysis (weakly positive).
+ = 25% inhibition of hemolysis (weakly positive).
— = Complete hemolysis (negative).

these reactions occurred with sera that were negative with the salt-solution and alcoholic extracts of Ascaris canis.

The results of complement-fixation tests in infestations with Tenia serrata and Dipylidium caninum are shown in Table 3. While the sera of a large number of dogs reacted positively with these antigens, particularly with the antigen of T. serrata, in surprisingly few were we able to find ova. The table includes only those dogs in the feces of which the ova or links were found.

TABLE 3

RESULTS OF COMPLEMENT-FIXATION TESTS IN INFESTATIONS WITH TENIA SERRATA AND DIPYLIDIUM CANINUM

Dog	Serum	Dose Serum, c.c.	Salt-Solution Extract T. Serrata	Alcoholic Extract T. Serrata	Salt-Solution Extract Dipylidium Caninum	Alcoholic Extract Dipylidium Caninum	Salt-Solution Extract Ascaris Canis	Alcoholic Extract Ascaris Canis	Salt-Solution Extract Trichocephalus Dispar	Salt-Solution Extract Strongylus Gigas	Alcoholic Extract T. Saginata	Serum Control
					Ova Tenia Serrata in Feces							
429	Inactive	0.1	+	+	—	—	—	—	—	—	—	—
434	Inactive	0.1	+	+	+	+	—	—	—	—	—	—
437	Active	0.1	—	—	—	—	0	—	—	—	—	—
320	Active	0.1	++	+++	—	—	—	—	—	—	—	—
459	Active	0.1	—	+++	—	—	—	—	±	—	—	—
					Ova of Dipylidium Caninum in Feces							
319	Active	0.1	—	—	—	—	—	—	—	—	—	—
320	Active	0.1	++	+++	++	+++	—	—	—	—	—	—
208A	Active	0.1	+	++++	—	—	+	+++	0	++	++++	—
173A	Active	0.1	+	+++	++	++++	—	—	—	—	+++	—

The results shown in Table 3 may be summarized thus:

1. Of 5 dogs infested with T. serrata, the sera of 3, or 60%, reacted positively with a salt-solution extract of T. serrata, and 4, or 80%, with the alcoholic extract of this parasite.

2. Of 4 dogs infested with Dipylidium caninum, the sera of 3, or 75%, reacted positively with the salt-solution and alcoholic extracts of this parasite.

3. Of the sera of 5 dogs infested with T. serrata, none reacted positively with an alcoholic extract of T. saginata, whereas the sera of 4 dogs infested with Dipylidium caninum reacted with this antigen.

The results of complement-fixation tests in infestations with Trichocephalus dispar (whip-worm) are shown in Tables 2 and 4. They may be summarized as follows:

Of 34 dogs infested with the whip-worm, the sera of all were negative with a salt-solution extract of this parasite. On account of the small size of the worm we were unable to obtain a sufficient number for the preparation of an alcoholic extract.

TABLE 4

RESULTS OF COMPLEMENT-FIXATION TESTS IN INFESTATIONS WITH TRICHOCEPHALUS DISPAR AND ANKYLOSTOMA CANINUM

Dog	Serum	Dose Serum, c.c.	Salt-Solution Extract T. Serrata	Alcoholic Extract T. Serrata	Salt-Solution Extract Diplydium Caninum	Alcoholic Extract Diplydium Caninum	Salt-Solution Extract Ascaris Canis	Alcoholic Extract Ascaris Canis	Salt-Solution Extract Trichocephalus Dispar	Salt-Solution Extract Strongylus Gigas	Alcoholic Extract T. Saginata	Serum Control
					Ova of Trichocephalus Dispar in Feces							
329	Active	0.1	—	++	—	—	—	—	—	—	—	—
330	Active	0.1	—	±	—	—	—	—	—	—	—	—
332	Active	0.1	—	—	—	—	—	—	—	—	—	—
335	Active	0.1	—	—	—	—	—	—	—	—	—	—
336	Active	0.1	—	—	—	—	—	—	—	—	—	—
318	Active	0.1	—	—	—	—	—	—	—	—	—	—
320	Active	0.1	—	—	—	—	—	—	—	—	—	—
319	Active	0.1	—	—	—	—	—	0	0	0	0	—
327	Active	0.1	—	—	—	—	—	—	—	—	—	—
194A	Active	0.1	—	++	—	—	—	—	—	—	—	—
216	Active	0.1	—	++++	—	—	—	—	—	—	—	—
400	Active	0.1	—	—	—	+	—	—	—	—	—	—
472	Active	0.1	—	—	—	+	—	—	—	—	—	—
453	Active	0.1	—	—	—	—	—	—	—	—	—	—
454	Active	0.1	—	++++	—	—	—	—	—	—	—	—
460	Active	0.1	—	—	—	—	—	—	—	—	—	—
462	Active	0.1	—	—	—	—	—	—	—	—	—	—
468	Active	0.1	—	—	—	—	—	—	—	—	—	—
470	Active	0.1	—	—	—	—	—	—	—	—	—	—
430	Active	0.1	—	—	—	—	—	—	—	—	—	—
					Ova of Trichocephalus Dispar and Ankylostoma Caninum in Feces							
329	Active	0.1	—	—	—	—	—	—	—	—	—	—
418	Active	0.1	—	—	—	—	—	—	—	—	—	—
492	Active	0.1	+++	—	—	±	+	—	—	—	—	—
461	Active	0.2	—	—	—	±	—	—	—	—	—	—
458	Active	0.2	—	—	—	—	—	—	—	—	—	—
474	Active	0.2	—	—	—	—	—	—	—	—	—	—
428	Inactive	0.1	++++	++++	—	—	—	—	—	—	—	—

As previously stated, the sera of a number of dogs reacted positively with the extracts of parasites the ova of which could not be found in the feces. Whether the antibody persisted in the body fluids after the parasite had been expelled, or whether we failed to find the ova altho the parasite was present, we are unable to decide. A third

possibility of much importance is the question of pseudo, or false, positive and non-specific reactions. All tests were conducted with one-quarter of the anticomplementary dose of the antigen in each case, and in all tests in which the serum control-tube showed an anticomplementary state of the serum, the results have been excluded. The results of complement-fixation tests in this series of dogs are shown in Table 5.

TABLE 5

RESULTS OF COMPLEMENT-FIXATION TESTS WITH SERA OF DOGS SHOWING NO OVA IN FECES

Dog	Serum	Dose Serum, c.c.	Salt-Solution Extract T. Serrata	Alcoholic Extract T. Serrata	Salt-Solution Extract Dipylidium Caninum	Alcoholic Extract Dipylidium Caninum	Salt-Solution Extract Ascaris Canis	Alcoholic Extract Ascaris Canis	Salt-Solution Extract Trichocephalus Dispar	Salt-Solution Extract Strongylus Gigas	Alcoholic Extract T. Saginata	Serum Control
224A	Active	0.1	−	++++	−	−	+	−	−	−	−	−
235A	Active	0.1	+	+++	−	++	−	−	+++	+++	−	−
242A	Active	0.1	−	+++	−	−	++	−	−	−	−	−
328	Active	0.1	−	−	−	−	−	−	−	−	−	−
333	Active	0.1	−	−	−	−	−	−	−	−	−	−
246A	Active	0.1	−	−	−	−	−	−	−	−	−	−
247A	Active	0.1	++	++++	−	−	−	−	−	−	−	−
285	Active	0.1	−	−	−	−	−	−	−	−	−	−
290	Active	0.1	−	−	−	−	−	−	−	−	−	−
298	Active	0.1	−	−	−	−	−	−	−	−	−	−
324	Active	0.1	−	−	−	−	−	−	−	−	−	−
325	Active	0.1	−	−	−	−	−	−	−	−	−	−
333	Active	0.1	−	−	−	−	−	−	−	−	−	−
390	Active	0.1	−	−	−	−	−	−	0	−	−	−
399	Active	0.1	−	−	−	−	−	−	0	−	−	−
493	Active	0.1	−	−	−	++++	++	++	−	−	−	−
448	Active	0.2	−	−	−	−	−	−	−	−	−	−
451	Active	0.2	−	−	−	−	−	−	−	−	−	−
455	Active	0.2	−	−	−	−	−	−	−	−	−	−
449	Active	0.2	−	−	−	−	−	−	−	−	−	−
461	Active	0.2	−	−	−	−	+	−	−	−	−	−
476	Active	0.2	−	−	−	−	−	−	−	−	−	−
463A	Inactive	0.1	++++	++++	−	−	+++	−	−	−	−	−
337	Inactive	0.1	++++	++++	−	−	−	−	−	−	−	−
431	Inactive	0.1	+	++++	−	−	−	+	−	−	−	−
433	Inactive	0.1	++	++++	−	−	−	+	−	−	+	−
435	Inactive	0.1	+++	++++	+	−	−	±	−	−	−	−
436	Inactive	0.1	++++	++++	−	+	−	−	−	−	−	−

Particular attention is drawn to the results of the complement-fixation tests with the inactivated sera. These were heated at 56 C. for one-half hour. As emphasized in our previous studies in non-specific complement fixation with normal rabbit and dog sera, heating sera at 56 C. greatly increases the antilytic activity and the percentage of non-specific complement-fixations. For this reason the majority of our tests have been conducted with fresh, active sera in dose of 0.1 c.c.

Heating at 62 C. tends to remove a large amount of the non-specific antilytic and complement-absorbing substances without materially depreciating the antibodies, and, when heating is necessary on account of thermolabil anticomplementary substances in serum, this degree of heat should be used.

TABLE 6

Summary of the Results of Complement-fixation Tests with Dog Sera and Extracts of Various Parasites

Antigen	Number of Dogs Tested	Reactions		Percentage Positive
		Positive	Negative	
Salt-solution extract of T. serrata	100	20	80	20
Alcoholic extract of T. serrata	100	42	58	42
Salt-solution extract Dipylidium caninum	100	4	96	4
Alcoholic extract of Dipylidium caninum	100	11	89	11
Salt-solution extract of Ascaris canis	100	17	83	17
Alcoholic extract of Ascaris canis	100	7	93	7
Salt-solution extract of Trichocephalus dispar (whip worm)	91	3	88	3.3
Salt-solution extract of Strongylus gigas	98	4	94	4
Alcoholic extract of T. saginata	99	2	97	2

As will be noted in Tables 2 to 4, many of the sera reacted positively with antigens of parasites the ova of which were not found in the feces. These results are summarized in Table 6, showing the percentage of positive reactions observed with the various antigens irrespective of whether the ova were or were not found in the feces.

In general the alcoholic extracts yielded a higher percentage of positive reactions than did the salt-solution extracts.

TABLE 7

Comparison of Results of Positive Complement-fixation Tests with Those of Positive Feces Examinations

Parasite	Percentage Positive Reactions		Percentage Positive Feces Examinations
	Salt-Solution Extracts	Alcoholic Extracts	
Tenia serrata	20	42	6
Dipylidium caninum	4	11	8.6
Ascaris canis	17	7	40
Trichocephalus dispar	3.3	0	43

Based on the examination of the feces of 100 dogs the following comparison has been made of the percentage of positive complement-fixations with the percentage of positive examinations for ova (Table 7). According to our results, it would appear that production of antibodies is most likely in infestations with the tapeworms (T.

serrata and Dipylidium caninum).   Antibodies are also produced in
infestations with Ascaris canis and the whip-worm, but in less degree;
particularly is this true in infestations with the whip-worm.

TABLE   8

Results of Complement-fixation Tests with Active Sera of Dogs Showing Parasitic Ova
in the Feces

Doses of Serum, c.c.	Antigens				Serum Control
	Salt Solution T. Serrata	Salt Solution Ascaris Canis	Alcoholic Extract Ascaris Canis	Salt Solution Dipylidium Caninum	
Dog 486.—Ascaris Canis and Ankylostoma Caninum					
0.01..........................	—	±	—	—	—
0.05..........................	—	++	—	—	—
0.1...........................	—	+++	+	—	—
0.2...........................	—	+++	++	—	—
Dog 502.—Ascaris Canis and Trichocephalus Dispar					
0.01..........................	—	—	—	—	—
0.05..........................	—	—	—–	—	—
0.1...........................	—	—	—	—	—
0.2...........................	—	—	—	—	—
Dog 505.—Ascaris Canis and Trichocephalus Dispar in Feces					
0.01..........................	—	—	—	—	—
0.05..........................	—	—	—	—	—
0.1...........................	—	—	—	—	—
0.2...........................	—	—	—	—	—
Dog 528.—Ascaris Canis in Feces					
0.01..........................	—	±	—	—	—
0.05..........................	—	++	—	—	—
0.1...........................	—	++++	+	—	—
0.2...........................	—	++++	++	—	—
Dog 319.—H. Serrata and Ascaris Canis in Feces					
0.01..........................	±	—	—	±	—
0.05..........................	++	+	—	+	—
0.1...........................	+++	++	—	++	—
0.2...........................	++++	++	+	++	—

SPECIFICITY OF THE COMPLEMENT-FIXATION

In view of the observation that the sera of dogs showing the pres-
ence of the ova of one parasite in the feces not infrequently reacted

positively with the antigens of other parasites, it is possible that the antibodies are not highly specific, and that in the dose of serum used (0.1 c.c.) the antibody for one parasite reacted with the extracts of other parasites in a general manner, as is not infrequently observed in complement-fixation with the immune sera against closely related micro-organisms such as the streptococci, diphtheria group, etc. We have studied this phase of the subject by conducting complement-fixation tests with descending doses of serum, and also by immunizing rabbits with the salt-solution extracts of various parasites and testing the specificity of the antibodies in a series of cross complement-fixation experiments.

Complement-fixation Tests with Descending Doses of Sera.—The results of 5 experiments with descending doses of the sera of 5 infested dogs with several antigens are given in Table 8. Ten such experiments were conducted, of which those given are examples of the results observed.

TABLE 9

Results of Complement-fixation Tests with the Inactivated Serum of Dog 24 Showing the Ova of T. Serrata in the Feces

Doses of Serum c.c.	Antigens					Serum Control
	Salt Solution T. Serrata	Alcoholic Extract T. Serrata	Alcoholic Extract Ascaris Canis	Alcoholic Extract of Syphilitic Liver	Cholesterinized Extract of Heart	
0.01	—	±	±	—	±	—
0.05	+	++	±	+	++	—
0.1	++	+++	+	++	++++	—
0.2	+++	++++	+++	++	++++	±

The experiments tabulated in Table 8 show that the antibody for Ascaris canis does not absorb complement with antigens of T.serrata and Dipylidium caninum. The antibody for T. serrata, however, absorbed complement in some degree with the antigen of Dipylidium caninum, and it is an open question as to whether these results may indicate a biologic relation between these parasites. The antibodies, therefore, when shown in the serum, appear to be highly specific, in so far, at least, as between those produced against Ascaris canis and those against T. serrata or Dipylidium caninum.

When inactivated sera (i. e. heated at 56 C.) are employed, and especially those of dogs showing well-marked tendencies toward nonspecific complement fixation, specificity is poorly marked, or entirely lost, as shown in Table 9.

Cross Complement-fixation Tests With Immune Sera.—A series of rabbits were immunized by repeated intravenous injections of salt-solution extracts of the following parasites: T. serrata, Dipylidium caninum, Ascaris canis, Trichocephalus dispar, Strongylus gigas.

TABLE 10

RESULTS OF CROSS COMPLEMENT-FIXATION TESTS WITH VARIOUS IMMUNE SERA (ACTIVE)

Active Serum, c.c.	Salt Solution T. Serrata	Salt Solution Dipylidium Caninum	Salt Solution Ascaris Canis	Salt Solution Trichocephalus Dispar	Salt Solution Strongylus Gigas	Serum Control
	Antigens					
With Tenia Serrata Immune Serum						
0.001	+++	—	—	—	—	—
0.005	++++	+	—	—	—	—
0.01	++++	+++	—	—	—	—
0.05	++++	++++	—	—	+	—
0.1	++++	++++	+	+	+	—
0.2	++++	++++	++	++	++	—
With Dipylidium Caninum Immune Serum						
0.001	—	—	—	—	—	—
0.005	+	+	—	—	—	—
0.01	+++	++	—	—	—	—
0.05	++++	+++	+	—	+	—
0.1	++++	++++	++	+	++	—
0.2	++++	++++	+++	++	+++	—
With Ascaris Canis Immune Serum						
0.001	—	—	—	—	—	—
0.005	—	—	+	—	—	—
0.01	—	—	++	—	—	—
0.05	—	—	++++	—	+	—
0.1	+	—	++++	—	++	—
0.2	+++	—	++++	—	+++	—
With Trichocephalus Dispar Immune Serum						
0.001	—	—	—	±	—	—
0.005	—	—	—	+	—	—
0.01	—	—	—	++++	—	—
0.05	—	—	+	++++	++	—
0.1	++	—	++	++++	++	—
0.2	+++	—	+++	++++	+++	—
With Strongylus Gigas Immune Serum						
0.001	—	—	—	—	++	—
0.005	—	—	—	—	++++	—
0.01	—	—	+	—	++++	—
0.05	—	—	++	—	++++	—
0.1	—	—	++++	—	++++	—
0.2	+	+	++++	—	++++	—

The material for these injections was prepared in the same manner as the salt-solution extracts used as antigens, except that they were preserved with 0.25% phenol instead of 0.5%.

Each rabbit received 6 injections at intervals of 5 days of 1, 2, 2, 3, 3, and 5 c.c., respectively, of each extract. One week after the last dose the animal was bled, the serum separated, and the tests conducted on the same and following days.

TABLE 11

RESULTS OF CROSS COMPLEMENT-FIXATION EXPERIMENTS WITH VARIOUS IMMUNE SERA
(HEATED AT 62 C.)

Heated Serum, c.c.	Antigens					Serum Control
	Salt Solution T. Serrata	Salt Solution Dipylidium Caninum	Salt Solution Ascaris Canis	Salt Solution Trichocephalus Dispar	Salt Solution Strongylus Gigas	
With T. Serrata Immune Serum						
0.001	+	—	—	—	—	—
0.005	++++	—	—	—	—	—
0.01	++++	+	—	—	—	—
0.05	++++	++	—	—	—	—
0.1	++++	+++	—	—	+	—
0.2	++++	+++	—	+	++	—
With Ascaris Canis Immune Serum						
0.001	—	—	—	—	—	—
0.005	—	—	±	—	—	—
0.01	—	—	+	—	+	—
0.05	—	—	+++	—	++	—
0.1	—	—	++++	—	+++	—
0.2	—	—	++++	—	++++	—
With Trichocephalus Dispar Immune Serum						
0.001	—	—	—	—	—	—
0.005	—	—	—	+	—	—
0.01	—	—	—	++	—	—
0.05	—	—	+	+++	—	—
0.1	+	—	+	++++	+	—
0.2	++	—	++	++++	+++	—
With Strongylus Gigas Immune Serum						
0.001	—	—	—	—	+++	—
0.005	—	—	—	—	++++	—
0.01	—	—	+	—	++++	—
0.05	—	—	+++	—	++++	—
0.1	—	—	++++	—	++++	—
0.2	—	—	++++	—	++++	—

All antigens were titrated before each experiment and used in one-quarter of their anticomplementary doses, as previously described.

These cross complement-fixation tests were conducted with fresh, active sera, sera heated at 56 C. for 30 minutes, and again with sera heated at 62 C. for 30 minutes.

The results observed with fresh, active sera are shown in Table 10; with sera heated at 62 C., in Table 11. They may be summarized as follows:

1. The active immune serum of T. serrata showed complement-absorption in best degree with its homologous antigen, and also to a well-marked extent with the antigen of Dipylidium caninum (Table 10). As shown in Table 8, similar reactions were observed with the serum of a dog infested with T. serrata, and in view of the wide morphologic differences between these tape-worms, we leave it an open question as to whether these reactions may be interpreted as indicating a biologic relation between them.

This relationship is again shown in Table 10, in the immune serum of Dipylidium caninum, which absorbed complement with the extract of T. serrata to the same degree as with its own or homologous antigen.

2. The active immune serum of Ascaris canis showed complement-absorption in best degree with its homologous antigen and to some extent with the antigen of Strongylus gigas (Table 10). These reactions may be interpreted as showing a biologic relation between these parasites, and this probable relationship is further indicated by the results observed with the immune serum against Strongylus gigas, which absorbed complement best with its own antigen and to a well-marked degree with the antigen against Ascaris canis (Table 10).

3. As shown in the tables, slight reactions frequently occurred with other antigens with the lower doses of serum. These reactions may have been due to non-specific complement fixation. Unfortunately, none of the rabbits used was subjected to preliminary complement-fixation tests before being accepted for the purposes of immunization, and we now believe that this precaution is of much importance when rabbit immune sera are being used in complement-fixation tests. Only those rabbits the sera of which are known to be free of non-specific complement-fixation bodies by reason of negative results in one or more preliminary complement-fixation tests should be selected for purposes of immunization. It is probable that the sera of rabbits used in preparing the immune sera shown in Table 10 would have shown non-specific complement fixation in doses of 0.1 and 0.2 c.c. before immunization.

4. Since heating rabbit and dog sera[16] at 62 C. tends to remove a portion or all of the non-specific complement-absorbing substances, we

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[16] Kolmer: Jour. Infect. Dis., 1916, 18, pp. 20, 27.

repeated these cross complement-fixation tests with sera heated at this temperature for half an hour. The results are shown in Table 11.

In this series of experiments the same biologic relation is indicated between T. serrata and Dipylidium caninum and between Ascaris canis and Strongylus gigas, while the reactions between the immune sera and other heterologous antigens are decreased or removed entirely as a result of heating the sera at 62 C.

On the other hand, heating these immune sera at 56 C. increased to an appreciable extent the tendency toward non-specific complement fixation. Accordingly, it would appear that complement-fixation tests with rabbit sera should be conducted with fresh, active sera, or, if heating is necessary to remove thermolabil anticomplementary bodies, with sera inactivated at 62 C. rather than at 56 C.

Of interest in this connection from the standpoint of applying results in complement-fixation tests in the diagnosis of intestinal parasitism of persons, is the question regarding the possibility of the sera of syphilitic persons reacting wth the extracts of intestinal parasites. We have conducted a number of complement-fixation tests with luetic sera and the extracts of parasites and found that some complement fixation occurred, especially with the alcoholic extracts. These results are not surprising in view of · the fact that the extracts contained lipoidal substances which were capable of absorbing complement with the syphilis reagin, as are other lipoidal substances.

We have tested the sera of 2 persons infested with T. saginata with these extracts of parasites from dogs, as well as with salt-solution and alcoholic extracts of T. saginata. The serum of one of these patients yielded strongly positive reactions with the extracts of T. saginata and T. serrata and to some extent with Dipylidium caninum, but none with the other antigens. The serum of the second person failed to fix complement with all antigens.

So far we have not had the opportunity of testing the serum of a syphilitic person infested with an intestinal parasite. It is probable, however, that if antibodies for both syphilis and parasite were present, these could be distinguished by a complement-fixation test conducted with constant doses of antigen and descending doses of serum, in which the group reaction would be lost in the smaller doses as 0.001, 0.005, 0.01, and 0.05 c.c. serum.

CONCLUSIONS

The results of this study are summarized in several subdivisions throughout the paper; here it may be stated that according to the results

of complement-fixation tests with the sera of infested dogs we have reason to believe that production of antibodies may occur after infestation of the intestines with the common parasites.

Production of antibodies was especially in evidence in infestations with tapeworms; to a less degree with the ascarides or round worms, and to a slight extent with the whip-worm.

These complement-fixations have tended to show a biologic relation between the tapeworms Tenia serrata and Dipylidium caninum and between Ascaris canis and Strongylus gigas, altho on account of the wide morphologic differences we leave it an open question; it is probable, therefore, that complement-fixation tests will not differentiate with the usual technic between related species of parasites, altho they may show the presence of a parasite.

Complement-fixation tests may be of value in the diagnosis of intestinal parasitism of man, and we are now making investigations in this field.

EPIDEMIOLOGY AND SYMPTOMATOLOGY OF AN OUT-BREAK OF SEPTIC SORE-THROAT IN WESTCHESTER COUNTY, NEW YORK *

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The first outbreak of septic sore-throat studied in America was the epidemic in Eastern Massachusetts described by one of us in 1912.[1] Since that time a number of other outbreaks have been reported: at Chicago, by Miller and Capps;[2] at Boston, by Coues;[3] at Baltimore, by Frost;[4] at Concord, N. H., by Mann;[5] at Cortland and Homer, N. Y. by North, White, and Avery;[6] at Middlebury College, Vt., by Eddy;[7] and at Rockville Center, N. Y., by Overton, Krumwiede, and Jaques.[8]

In most of these cases the disease was spread chiefly by unpasteurized or imperfectly pasteurized milk. It has of course been recognized by all who have studied this disease that it may be, and is, transmitted not only by milk, but also by contact. In the Eastern Massachusetts outbreak the milk epidemic in Boston and its suburbs was preceded by prosodemic spread in Hudson, Marlboro, and Southboro; while at Baltimore the milk epidemic was followed by a prosodemic outbreak. Yet the emphasis on milk as a causal factor has been almost overwhelming in all these cases. In view of this fact the present outbreak, chiefly prosodemic in nature, may be of interest to epidemiologists.

THE WESTCHESTER COUNTY OUTBREAK

Septic sore-throat has been a reportable disease in the state of New York since May 1, 1914, but its reporting is still very incomplete. When it appeared in March, 1915, that there was an unusual prevalence of this

[1] Jour. Infect. Dis., 1912, 10, p. 73.
[2] Jour. Am. Med. Assn., 1912, 58, p. 1848.
[3] Am. Jour. Pub. Health, 1912, 2, p. 419.
[4] Pub. Health Rep., 1912, 27, p. 1889.
[5] Jour. Infect. Dis., 1913, 12, p. 481.
[6] Ibid., 1914, 14, p. 124.
[7] Bull. Vermont State Board of Health, 1914, 14, p. 25.
[8] Bull. New York State Dept. of Health, 1914, 9, p. 230.

disease in certain municipalities of Westchester County, a special study was undertaken to determine its actual extent and etiology.

Cards calling for the name and address, age and sex of each patient, the date of first visit, and the principal symptoms noted, were printed and distributed to all the physicians in the city of Mt. Vernon and the villages of White Plains, Bronxville, and Tuckahoe, the communities affected. The list of symptoms in regard to which information was specifically requested on the cards was as follows: throat much reddened, false membrane, high temperature (above 102 degrees), prostration, swelling of tonsils, quinsy, cervical glands involved, ears involved, relapses, rheumatism, erysipelas, nephritis. Endocarditis was omitted by an oversight, but was written in on the cards in a few instances. We desire to express our great appreciation of the cooperation of the physicians of Mt. Vernon, White Plains, Bronxville, and Tuckahoe which made this study possible. They responded most cordially to our request for this information, and the returns obtained are believed to be fairly complete.

In all, we obtained reports of 905 cases of septic sore-throat in the three communities from January to May: 556 cases in Mt. Vernon (population, 38,000); 193 cases in Bronxville and Tuckahoe (combined populations about 5,000); and 156 in White Plains (population 19,000).

The distribution by weeks and according to milk supply (to which reference will be made later) is shown in Table 1 and Fig. 1. The

TABLE 1

Cases of Septic Sore-throat Summarized by Communities, by Weeks, and by Milk Supplies

Dates	Mt. Vernon Milk Supply		Bronxville and Tuckahoe Milk Supply		White Plains Milk Supply		Total Milk Supply	
	Dairy "A"	Others	Dairy "A"	Others	Dairy "A"	Others	Dairy "A"	Others
Before Feb. 15..........	4	20	17	6	3	1	24	27
Feb. 15-21	44	22	8	9	10	3	62	34
Feb. 22-28	84	43	31	14	11	5	126	62
Mar. 1-7	71	43	19	22	10	11	100	76
Mar. 8-14	38	44	12	22	10	8	60	74
Mar. 15-21	30	22	9	6	5	3	44	31
Mar. 22-28	15	20	6	6	6	6	27	32
Mar. 29-Apr. 4..........	3	10	...	4	1	4	4	18
Apr. 5-11	7	11	...	1	7	7	14	19
Apr. 12-18	2	8	...	...	3	1	5	9
Apr. 19-25	...	2	1	...	2	2	3	4
Apr. 26-May 2...........	2	2	...	...	...	10	2	12
Later or unknown date	...	9	...	...	18	9	18	18
Totals	300	256	103	90	86	70	489	416

chief prevalence of the disease in all three districts was between February 15 and March 29 with a maximum in the week beginning February 22. The fact that the cases occurred in considerable numbers for a period of 6 weeks seems to point toward a spread in prosodemic fashion, rather than in the form of a milk epidemic, since milk epidemics of this disease usually show an overwhelming preponderance of cases during a single week or fortnight.

SEX INCIDENCE

Information as to the sex of the patients is presented in Table 2. Of the 893 cases in which sex was reported, 517, or 58%, were females. This corresponds closely with the results reported in most of the other outbreaks. Of the Boston (milk) cases in Eastern Massachusetts 71% were females and of the Hudson (prosodemic) cases 45%; but at Baltimore, 58% of the cases were females, at Cortland and Homer 57%, at Concord 58%, and at Rockville Center 59%.

TABLE 2

Cases of Septic Sore-throat in Westchester County Summarized According to Sex

	Male	Female	Unknown
Mt. Vernon	240	306	10
Bronxville and Tuckahoe..................................	75	117	1
White Plains	61	94	1
Total	376	517	12

AGE INCIDENCE

The age incidence of 724 cases (Westchester County) in which the age was stated is shown in Table 3; and the percentage of the total number of cases of known age at each age period in this outbreak as compared with 4 others in Table 4.

TABLE 3

Cases of Septic Sore-throat in Westchester County Summarized According to Age

Age Not Stated	Under 11 yr.	11-20 yr.	21-30 yr.	31-40 yr.	41-50 yr.	51-60 yr.	Over 60 yr.
181	181	119	146	153	81	30	14

The Baltimore outbreak was marked by a heavy prevalence of cases (53%) at ages under 10 years, and the Concord outbreak shows

an excessive incidence at ages from 21 to 30. The other three sets of figures indicate a fairly even distribution of cases over the various age periods, as in the Eastern Massachusetts outbreak.

TABLE 4

Age Distribution of Cases of Septic Sore-throat in Various Outbreaks

Outbreak	Percentage of Total Number of Cases of Known Age at Each Age Period						
	Under 11 yr.	11-20 yr.	21-30 yr.	31-40 yr.	41-50 yr.	51-60 yr.	Over 60 yr.
Westchester County.........	25	16	20	21	11	4	2
Baltimore....................	53	12	15	10	6	2	3
Cortland-Homer.............	15	16	18	19	13	8	12
Rockville Centre.............	29	13	22	20	9	3	5
Concord*....................	.. 18		42	22	..	.. 18	

* Town population excluding cases in large schools.

SYMPTOMATOLOGY

The data obtained by us in regard to symptomatology are somewhat more extensive than those previously published. The relative proportion of cases in which each symptom was noted is indicated in Table 5, according to milk supply (reference to this point will be made later), and in Table 6, according to age.

TABLE 5

Symptomatology of Septic Sore-throat in Westchester County

Symptom	Percentage of Total Cases Showing Each Symptom		
	Customers of Dairy "A"	Other Cases	Total Cases
Reddening of throat..........................	83	84	83
False membrane.............................	20	20	20
High temperature............................	41	32	37
Prostration.................................	48	45	47
Tonsils enlarged.............................	62	58	60
Quinsy.....................................	12	6	9
Glands involved.............................	53	46	50
Ears involved...............................	8	6	8
Relapses....................................	7	7	7
Rheumatism.................................	12	10	11
Erysipelas..................................	2	2	2
Nephritis...................................	3	2	3
Endocarditis................................	0.4	0.4	0.4

Reddening of the throat was almost universal, being reported in 83% of 905 cases. Enlargements of tonsils and glands were noted in about half the cases (60% and 50% respectively). Marked prostration was reported in 47% of the cases, and a temperature of over 102 degrees in 37% of cases. Formation of false membrane was less com-

mon than is often the case in such outbreaks—observed in only 20% of our cases. Among the rarer complications were quinsy (9%), involvements of the ears (8%), nephritis (3%), erysipelas (2%), and endocarditis (0.4%). The last figure is perhaps too low, since no place was provided for this entry on our card. Seven percent of all cases suffered from relapses.

TABLE 6

Symptomatology of Septic Sore-throat at Different Age Periods in the Westchester County Outbreak

Symptom	Percentage of Cases at Each Age Period Showing Each Symptom						
	Under 11 yr.	11-20 yr.	21-30 yr.	31-40 yr.	41-50 yr.	51-60 yr.	Over 60 yr.
Reddening of throat........	87	86	93	94	86	93	93
False membrane............	20	24	20	33	15	20	7
High temperature...........	44	37	38	46	44	50	57
Prostration.................	42	43	56	64	58	80	64
Tonsils enlarged............	60	68	73	69	61	63	57
Quinsy.....................	0.6	5	16	13	9	33	0
Glands involved............	63	58	51	48	44	50	14
Ears involved..............	15	6	8	9	3	7	7
Relapses...................	6	5	8	8	15	16	0
Rheumatism................	2	7	10	21	26	20	7
Erysipelas.................	0	1	0	1	1	7	14
Nephritis..................	0.6	3	3	3	5	10	7
Endocarditis...............	0.6	1	1	0	0	3	0

The distribution of symptoms by ages (Table 6) is of considerable interest. It will be noted that the symptoms which may be held to measure the general severity of the disease, such as high temperature, prostration, relapses, rheumatism, erysipelas, and nephritis, tend to increase in frequency with advancing years; on the other hand, involvements of the glands and ears are most common in young persons.

THE CAUSE OF THE OUTBREAK

Communicable diseases may be considered for practical purposes to be spread in 3 ways—by contact, by articles of food and drink, and by insects. Septic sore-throat has never been connected with an insect carrier, and the season and the habits of the affected population make it unnecessary to consider this factor in Westchester County. So far as water and food supplies other than milk and cream are concerned, none of these, so far as we are aware, is distributed to the three sections of Westchester County affected which is not also distributed elsewhere. The inquiry very soon, therefore, narrowed itself down to the two factors, contact and milk.

The general distribution of the disease in time, as noted above, was such as to suggest prosodemic spread by contact rather than an epidemic due to milk. There is, however, one large dairy company, and only one, which supplies a large portion of the population of all three of the communities affected; and the possible connection of this dairy with the outbreak demanded study.

Through the courtesy of the managers of the dairy company in question we were given free access to its records. Each address from which a case of septic sore-throat had been reported was compared with the delivery route books to see whether milk was delivered there by the "A" company or not. This method, the same one which was pursued in the Boston outbreak, is of course open to obvious errors in individual cases. There are, on the one hand, members of families in which milk is bought who do not drink it; and, on the other hand, there are those who may have drunk infected milk while visiting at the homes of others. On a broad scale and for comparative purposes, however, such a study yields all the data really necessary; and its results are probably as near the truth as those based on the memories of individuals as to whether they did or did not drink milk during a specified period.

The "A" company furnishes about 45% of the total milk supply of Mt. Vernon, Bronxville, and Tuckahoe, and 54% of the cases of septic sore-throat were among its customers; while in White Plains, where the company supplies 70% of the milk, there were only 55% of the septic sore-throat cases in the houses which it served. These figures seem to furnish clear evidence that the general prevalence of the disease was not due to milk in any large degree, and confirm the inference, drawn from the dates of incidence, that it was the result of spread in prosodemic fashion by ordinary contact.

A study of the time distribution of the "A" cases and the other cases in Table 1 and Figure 1 suggests, however, that the problem may not be an altogether simple one. Between February 15 and March 8, the period when the outbreak was at its height, 63% of the cases in Mt. Vernon, Bronxville, and Tuckahoe were in households served by the "A" dairy, while before and after that period only 44% were in such households. In White Plains during the same three weeks 62% of the cases were in households receiving "A" milk against 52% for the period before and after. Figure 1 shows these facts in graphic form. The fact that between February 15 and March 8 the "A" households

Fig. I

TIME INCIDENCE SEPTIC SORE THROAT CASES
(WESTCHESTER COUNTY)
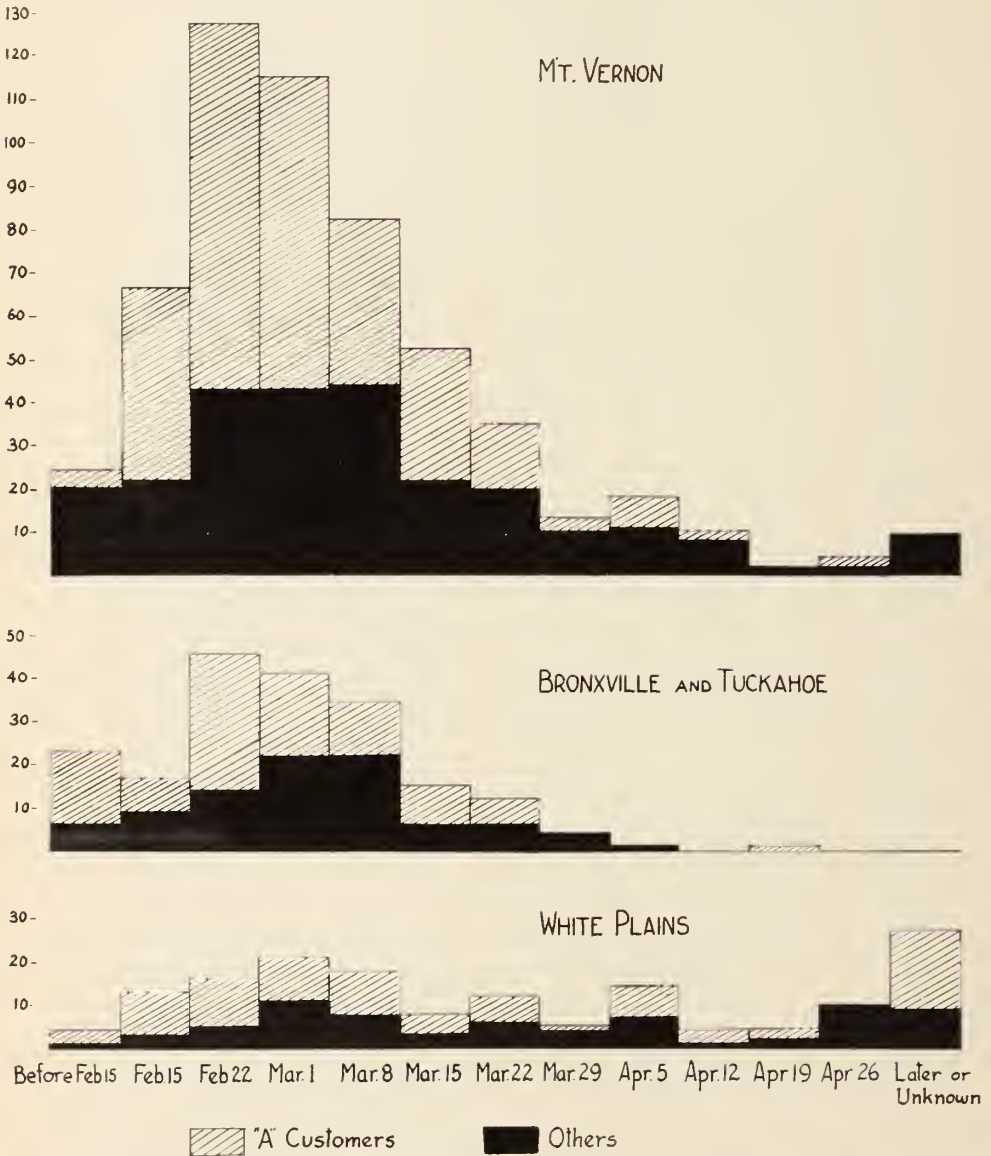
Fig. II

FAMILY INCIDENCE IN SEPTIC SORE THROAT
SPREAD BY MILK AND BY CONTACT

Percentage of Families Having 1, 2, 3, or more Cases
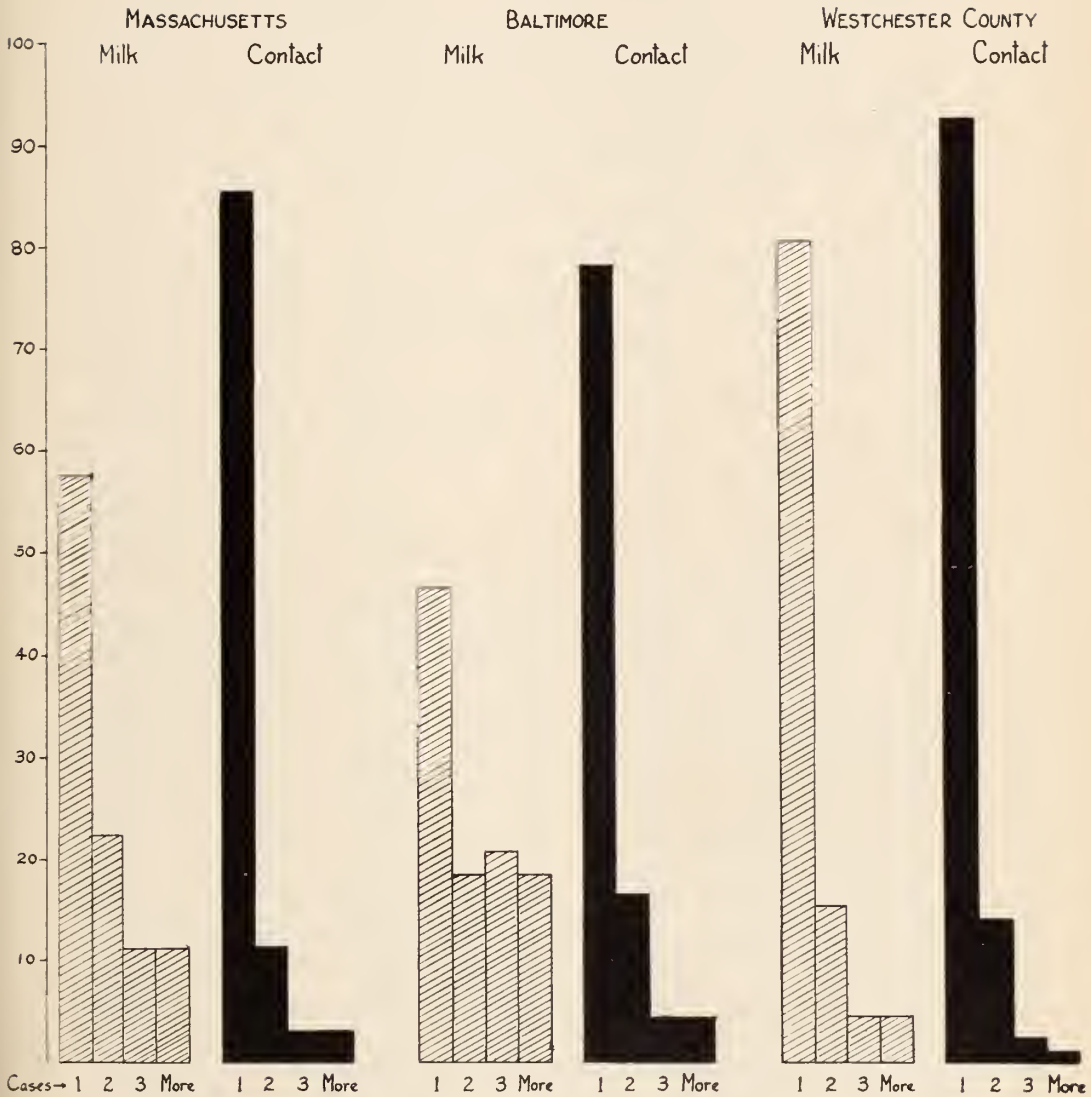
Fig. 2.—The cross-hatched cases in the Westchester outbreak are among "A" customers. Some of these cases, but by no means all, were presumably due to milk infection.

suffered proportionately much more than before and after this period suggests that a small milk or cream epidemic may at this time have been superimposed upon a general prosodemic spread. This hypothesis is strengthened by a comparison of certain characteristics of the groups of cases in the "A" households with those of cases in the other families.

INTENSITY OF FAMILY INCIDENCE

Table 7 shows the general intensity of family incidence in the "A" households and in the other households, and in Table 8 these data are compared with those reported in other outbreaks.

TABLE 7

FAMILY INCIDENCE OF CASES OF SEPTIC SORE-THROAT IN WESTCHESTER COUNTY

Number of Cases in Family	Families		Cases	
	"A" Households	Others	"A" Households	Others
1....................	273	330	273	330
2....................	53	29	106	58
3....................	13	8	39	24
4....................	4	1	16	4
5....................	5	....	25	....
6....................	1	....	6	....
12....................	2	....	24	....
Totals..........	351	368	489	416

TABLE 8

FAMILY INCIDENCE OF CASES OF SEPTIC SORE-THROAT IN VARIOUS OUTBREAKS

Outbreak	Percentage of Total Families Affected Having			
	1 Case	2 Cases	3 Cases	4 or More
Boston*..	56	22	11	11
Hudson, Mass.†....	83	11	3	3
Baltimore, dairy customers*...............	45	18	20	18
Baltimore, others†..........................	76	16	4	4
Westchester, dairy customers*.............	78	15	4	4
Westchester, others†.......................	90	14	2	1

* Wholly or partly milk infection.
† Wholly contact infection.

Table 8 and Fig. 2 bring out in a striking manner the fact that where septic sore-throat is milk-borne, the proportion of multiple cases in a family is much greater than where the disease is spread by contact, altho the absolute intensity of infection varies with the virulence of the organism in different outbreaks, having been more severe in Baltimore and comparatively mild in the present outbreak. The same observation was made by Miller and Capps in the Chicago outbreak. Among the users of the infected milk there were 2.3 cases to a family, while among

Fig. III
SEVERITY OF SYMPTOMS

Reddening of Throat — 84% / 83%
Tonsils Enlarged — 58% / 62%
Glands Involved — 46% / 53%
Prostration — 45% / 48%
High Temperature — 32% / 41%
Pseudo-membrane — 20% / 20%
Rheumatism — 10% / 12%
Quinsy — 6% / 12%
Ears Involved — 6% / 8%
Relapses — 7% / 7%
Nephritis — 2% / 3%
Erysipelas — 2% / 2%
Endocarditis — 0.4% / 0.4%

'A' Customers   Others
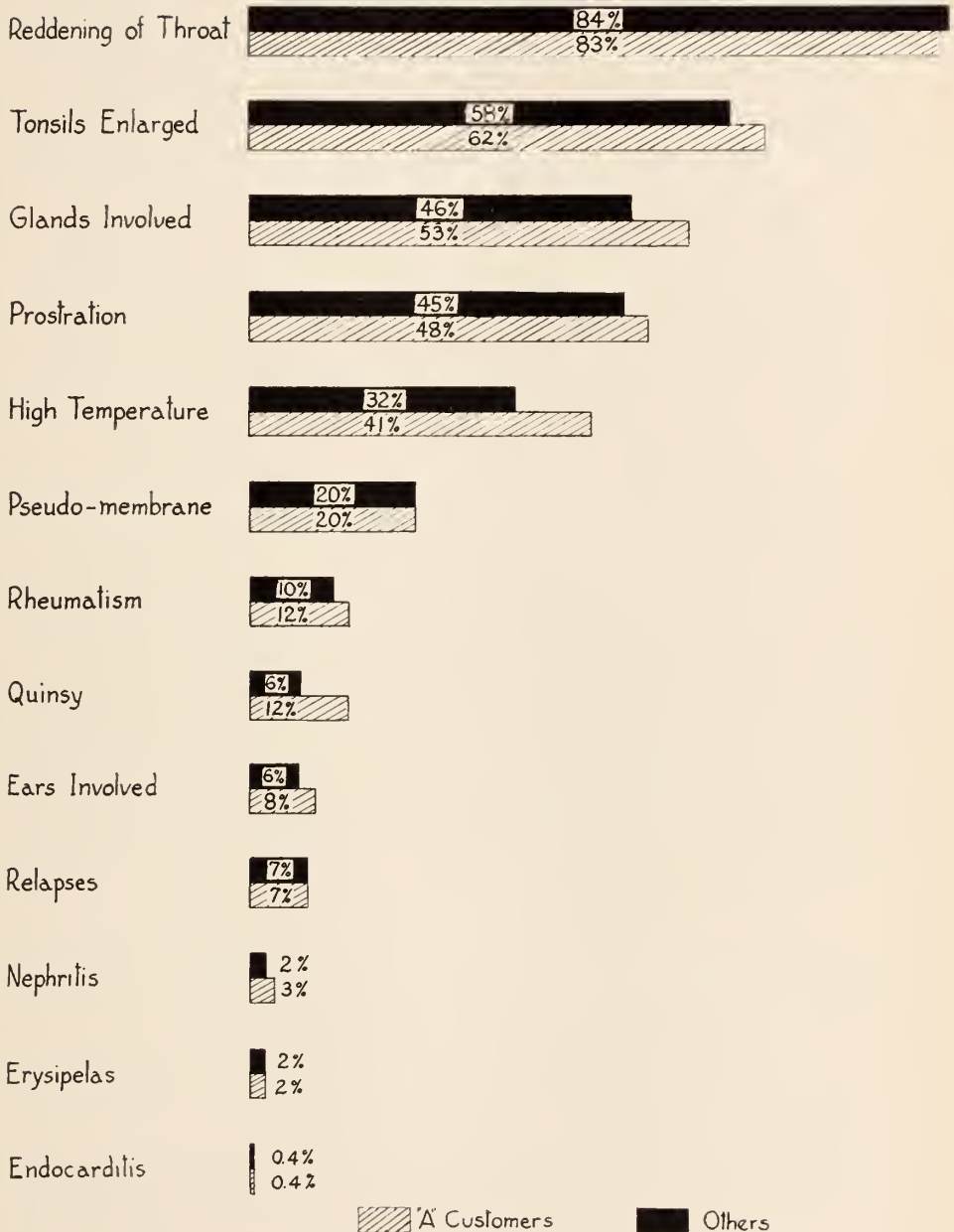
other persons the average was 1.2 cases. In the milk outbreak at Concord there was an average of 2 cases to a family. The average for our "A" households was 1.4 cases to a family and for the others 1.1 cases, this again suggesting that a part, altho only a small part, of the outbreak was due to milk or cream infection. It is of interest to note that of 46 instances in which 2 cases developed in a single family on the same day, 33 were in families supplied with "A" milk.

SEVERITY OF THE DISEASE IN RELATION TO MILK SUPPLY

In the Massachusetts outbreak of 1911 the septic sore-throat spread by milk in Boston and vicinity was much more severe than the prosodemic infection in Hudson and adjoining towns from which the milk infection was originally derived. In the Baltimore outbreak Frost noted that the symptoms in milk cases were much more pronounced than those of persons infected by contact. We have analyzed our data from this standpoint in Table 5 and in Fig. 3.

It will be noted that many of the symptoms, notably high temperature, tonsillar and glandular enlargement, quinsy, infection of the ears, rheumatism, and nephritis, were distinctly more common in the "A" households than in the others—another item of circumstantial evidence in favor of the view that the "A" milk or cream did really play a certain part in the spread of septic sore-throat in Westchester County. Careful inquiry, however, failed to locate any frankly infected individual who had come in contact with "A" milk at any stage of its production or distribution. Both milk and cream were mixed supplies derived from a large number of farms and both were delivered raw. Milk infection can of course account only for a small proportion of the entire outbreak, the great majority of cases being undoubtedly of prosodemic origin.

CONCLUSIONS

An outbreak of 905 cases of septic sore-throat occurred in 4 neighboring communities of Westchester County, New York, in the spring of 1915, reaching its maximum between February 15 and March 22.

The distribution of cases of the disease over a long period of time and the absence of any common food or milk supply for a majority of the cases indicates that it was spread, for the most part, by contact and in prosodemic fashion.

The fact that at the height of the outbreak (February 15 to March 8) there was an increase in the proportion of cases in households sup-

plied by a certain milk dealer, that the family incidence in these households was unusually high, and that the severity of the disease in them was accentuated, indicates that a small milk or cream epidemic was then superimposed on the general prosodemic spread of the disease.

Septic sore-throat tends to affect females much more than males. In the five outbreaks which have been reported at Baltimore, Cortland and Homer, N. Y., Concord, N. H., Rockville Center, N. Y., and Westchester County, N. Y., 57-59% of the cases have been females.

Septic sore-throat usually affects all ages without marked preference.

In the Westchester County outbreak 83% of the cases showed reddening of the throat, 60% tonsillar involvement, 50% glandular involvements, 47% prostration, 37% a high temperature (over 102 degrees), 20% false membrane, 11% rheumatism, 9% quinsy, 8% ear infection, 7% relapses, 3% nephritis, 2% erysipelas ,and 0.4% endocarditis.

High temperature, prostration, relapses, rheumatism, erysipelas, and nephritis were symptoms more common among the old; involvements of glands and ears, among the young.

When septic sore-throat is spread by contact, multiple cases in a household are less numerous than when it is spread by milk.

Septic sore-throat is generally more severe when milk-borne than when spread in prosodemic fashion.

THE VALUE OF VIRULENT SALT SOLUTION IN THE PRODUCTION OF ANTIHOG-CHOLERA SERUM BY THE INTRAVENOUS METHOD [*]

Robert Graham and L. R. Himmelberger

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Craig[1] first suggested the use of virulent salt solution in the manufacture of antihog-cholera serum. Its use also received attention from Robbins.[2] Their work indicates clearly that salt solution made virulent by passing through the peritoneal cavity of virus pigs can be injected subcutaneously into immune hogs in sufficient quantities to produce a potent antiserum. The intravenous method of serum-production is more widely used, but up to this time no systematic attempt has been made to use virulent salt solution in the course of hyper-immunizing by this method.

The necessity of employing large amounts of virus in the production of antihog-cholera serum of high potency by the subcutaneous method has prevented its general adoption. The cost of producing antihog-cholera serum by the intravenous method of injection, aside from other desirable features, is much less. This is quite generally conceded inasmuch as it requires about one-half the volume of blood virus if an immune animal is to be rendered hyperimmune by an initial dose of virus. Since the cost of serum is dependent upon the cost of the virus, any method which will increase the quantity of virulent material to be obtained from a single virus pig is therefore a factor in reducing the cost of serum. No doubt large quantities of virus are retained in the bodies of virus-producing animals, which under the present method are cremated. Attempts to remove the virus from the musculature and organs in sufficient quantities to render an animal hyperimmune following injection have not been consistently successful thus far.

There exist no means of determining the degree of virulence of a given virus other than that indicated by illness following its injection into susceptible shoats and by the potency of the antiserum produced by its use in hyperimmunization. If by the use of virulent salt solution

[1] Bull. Pardue Experiment Station, 140.

[2] Jour. Infect. Dis., May, 1913, 12, p. 335.

in the intravenous method an antiserum of proven protective power can be produced, but little objection can be raised to the use of this method.

The work we wish to describe was conducted according to the ideas of Craig and of Robbins, departing from their technic in that we used the salt virus intravenously. They found, after extensive experiments, that virulent salt solution of hyperimmunizing value was best obtained, for the subcutaneous method, by the intraperitoneal injection of 25 to 30 c.c. of 0.85% salt solution per pound weight of the virus pig. This was allowed to remain in the peritoneal cavity for 5 to 6 hours. In our work 25 c.c. per pound weight of sterile 0.9% salt solution were injected into the peritoneal cavity of virus pigs at least 5 hours before killing, when the salt solution was removed aseptically. By the injection of salt solution the volume of blood obtained is considerably increased, approximately 10 to 20%. The salt solution is recovered in 40 to 70% of the volume injected. Table 1 indicates the approximate increase in the amount of virus obtained, with the relative amounts of salt solution administered and regained in a series of 11 pigs, the figures referring to quantities in cubic centimeters.

TABLE 1

Increase in Amount of Blood Virus Obtained by Intravenous Injection of Salt Solution, and the Relative Amounts of Salt Solution Injected and Redrawn

Pig (Weight 100-110)	Virus Blood Drawn	Salt Solution	Salt Solution Redrawn	Estimated Approximate Increase in Blood Virus	Total
1................	1,800	2,500	1,200	200	3,000
2................	1,600	2,500	1,600	400	3,200
3................	2,150	2,500	1,600	550	3,750
4................	1,800	2,500	1,100	600	2,900
5................	1,950	2,500	1,600	350	3,550
6................	2,150	2,500	2,000	550	4,150
7................	2,000	2,500	1,950	400	3,950
8................	2,400	2,500	1,650	800	4,050
9................	1,800	2,500	1,700	200	3,500
10................	1,900	2,500	1,500	300	3,400
11................	2,000	2,500	None	400	2,000
Total........	21,550	27,500	15,900	4,750	37,450

The salt virus and blood virus were first used for hyperimmunizing in 3 different proportions, namely:

Blood Virus		Salt Virus	Amount Injected
1 part	+	3 parts	7 c.c. per lb.
2 parts	+	2 parts	7 c.c. per lb.
3 parts	+	1 part	7 c.c. per lb.

The antisera yielded by all three of the animals indicated, proved to be protective when tested on susceptible shoats. Table 2 gives the results of the test on the sera produced in this way.

TABLE 2

RESULTS OF TESTS WITH ANTISERA MADE WITH VARYING AMOUNTS OF SALT VIRUS AND BLOOD VIRUS

Weight of Pig	Dose Virus, c.c.	Dose Serum, c.c.	Temperatures — May															Remarks
			15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
50	2	20	102.1	102.3	102.4	102.3	103	103.1	102.3	102	103	102.3	102.2	102.4	...	...	...	}Serum produced with ½ salt virus intravenously
60	2	20	102	102.1	103	103.1	103	102.2	102.3	101.1	102.1	103	103	103	...	...	...	
75	2	20	102	102.4	102.4	102.1	102.1	102.1	102	102	102	102	102	102	...	...	...	}Serum produced with ½ salt virus subcutaneously
60	2	20	102.2	102.3	103.2	103	102	101	102	102	103.1	102.2	102.2	102.4	...	...	...	
60	2	20	102.4	102.3	102.4	102	102	101.1	102.1	102.1	102	102	103	102.3	...	...	...	}Serum produced with ¾ salt virus intravenously
60	2	20	103.1	103.4	103.3	103	103.4	102.2	102.1	102.2	102.4	102.1	102.2	102	...	...	...	
65	2	..	102	102.1	102.4	102	102	102.3	103.4	103.5	103.1	103.4	104.3	106.2	106	105	105.4	}Controls. Chronic hog-cholera
60	2	..	101.3	102	102.1	102.4	102.3	102.2	103.1	104.1	104.2	104.3	104.4	104.5	104.7	104.5	104.6	

In subsequent experiments the following method was used: Immunes weighing from 150 to 300 pounds were injected with a mixture of equal parts of virus and virulent salt solution at the rate of 7 c.c. per pound body weight. Some tests were carried out in which the salt virus and blood virus were mixed without particular attention to the volume of either. The mixtures used varied from straight blood virus, in those pigs in which by error the salt solution was not aseptically drawn or was otherwise discarded, to equal proportions of each, but in most cases the salt mixture used was 60% blood virus and 40% salt virus. The serum resulting from hyperimmunizing in these proportions protected susceptible shoats against 2 c.c. of virus. The use of salt virus and blood virus as drawn from the virus pig in the routine production of antihog-cholera serum by the intravenous method is more practical than the use of other proportions, as it eliminates handling the salt and blood virus separately.

The antisera obtained from hyperimmunes of one series were mixed. To determine the potency of this mixture, we tested it on susceptible shoats weighing 50 to 100 pounds, as described in Table 3. (Dr. K. L. Pontius had charge of the testing of all experimental sera.)

TABLE 3

RESULTS OF TESTS FOR POTENCY OF ANTISERA

TEST 1

Weight of Pig	Dose Virus c.c.	Dose Serum c.c.	Temperatures April						May					Remarks
			25	26	27	28	29	30	1	2	3	4	5	
100	2	30	102	102.1	102.2	102.1	102.2	102	103	104	104	103	102.3	Serum manufactured by hyperimmunizing immune hogs with ½ salt and ½ blood virus
100	2	30	102.3	102.3	102.3	102.1	102	102.4	102.5	102	103.4	103.2	102.4	
100	2	40	102.4	102.3	102.4	102.3	102.2	102.3	103.2	103.3	103.4	102.4	102.2	
100	2	28	103	103	102	102	102.1	102.2	103.5	103.6	104	103.4	103	
100	2	50	102.2	102.1	102.2	102.1	102	102.4	102	102	103.2	103.1	102.5	
100	2	50	101.3	102	102.3	102.1	102	102.4	102	103	103.1	102.2	102.4	
100	2	..	103.4	102.4	102.3	102.3	102.4	103.1	104.2	104.3	104.4	104.6	105	Died } Controls
100	2	..	102.2	103.3	103.4	104	104.2	104.3	104.4	104.4	104.5	105	105	Died }

TEST 2

Weight of Pig	Dose Virus c.c.	Dose Serum c.c.	Temperatures April								May					Remarks
			23	24	25	26	27	28	29	30	1	2	3	4	5	
100	2	25	102	102.1	102	103	103.3	102.2	102.2	102	103	103.2	103	102.3	102.2	Serum manufactured by hyperimmunizing immune hogs with salt virus and blood virus mixed just as drawn from cholera pigs
90	2	25	102.1	101.2	101.3	102.4	102.4	102.3	102	102.3	102.4	102.4	102.4	102.2	102	
100	2	20	101.1	102	102.3	102	103	102	102.1	102.6	102.5	102.4	103	103.1	102	
98	2	20	102.2	102.4	102.2	103.2	103	103.2	102	102.4	102.4	103.1	103	102.4	102	
80	2	15	102	102	102.2	104	104	103	103.1	103	103.2	103.1	103.3	102.3	102	
76	2	15	101.2	102.1	102.1	103.4	103.2	102.4	102.3	102.4	103.1	103.4	103.2	102.4	102.3	
75	2	..	102.1	102.3	102.4	104.3	104.2	104.1	104.2	104.5	Died............					} Controls
75	2	..	102.4	102.3	102.3	104.2	104.1	104	105.4	106	105.1	Died.........				}

TEST 3

Weight of Pig	Dose Virus c.c.	Dose Serum c.c.	Temperatures May															Remarks
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
58	2	25	102	102	102	102.1	102.2	102.2	102.2	102	102.2	103	103	103.2	104.2	104	104	Serum manufactured by hyperimmunizing immune hogs with salt virus and blood virus mixed as drawn from cholera pigs
60	2	25	102.2	102.3	103	102.4	103	102.4	102.3	103.1	102.2	103.2	103.2	103.1	104	104.2	104.2	
60	2	20	103	102.1	102.2	103	103	103.1	103	102	102.4	102.1	102.1	104.4	103.1	103.5	103.3	
60	2	20	102.1	102.5	102.3	102.2	102.2	102	102.2	102.2	102.1	103.4	103.4	103.4	103.5	104	104	
62	2	15	103	103	103	103.1	103	103.5	103.4	102.3	102.4	102.2	102.3	102.4	103.4	103.5	103.4	
65	2	15	102.2	102.1	103.1	102.3	102.4	102.2	102.1	102.1	102	104.1	103.1	103.5	104	103.2	104	
62	2	..	102.3	102.4	103.3	103.4	103.5	103.6	104.3	104.4	Died..................							} Controls
62	2	..	102.4	103.2	103.4	103.6	103.6	104	104.2	103.1	Died..................							}

Table 3.—*Continued*

Test 4

Weight of Pig	Dose Virus c.c.	Dose Serum c.c.	Temperatures — May																				
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
60	2	15	102	102.2	102.3	102.2	102	102	102	102	102.2	102.3	102.3	102.1	102.3	104.2	104	103.5	103.4	102.5	102.4	101	101
65	2	15	102	102.1	103.4	103.2	103.1	103	103	103.1	102.1	102.2	102.4	102.3	102.1	104.2	103.2	102.4	102.4	103	103	100	101
54	2	20	103	102	103	102.4	102.2	102.5	102.4	102	102.4	103.2	103	102	104.6	103.5	103.1	104	104	104	102	102	102
55	2	20	102.1	102	102.4	103	103	103	102	102	102.2	102.4	103.1	102.4	103.4	103.4	104.4	104.3	104.2	104.1	103.2	102.1	102.1
60	2	25	102	102	103	103.1	102.4	102.5	102.1	102.2	102.1	102.1	102.2	102	103.4	104.2	103.2	103.6	104.1	103.2	103	102	102
65	2	25	102.3	102.2	102.1	102.4	103.4	102	102.4	102	102.4	102.3	102.2	102.3	103.4	103.1	103.3	103	103	103	102.4	103.2	101.4	102
54	2	..	102.1	101.6	102	103	104	104.1	105.6	Died													
68	2	..	103.3	102.4	102.1	103.5	103	104.2	106	Died													

Controls (rows 7–8).

Serum manufactured by hyperimmunizing immune hogs with salt virus and blood virus as drawn from cholera pigs.

The objection which may possibly be raised to the use of virulent salt solution is that the blood virus is weakened by dilution. This is possible, but in view of the fact that the serum produced by hyperimmunizing with mixtures of salt virus and blood virus protects susceptible shoats, such objections are not fundamental. In laboratories which make a practice of testing all serum before it is distributed for use in the field, the salt solution method has proved a perfectly safe and an economical procedure; that is, in the Indiana and Michigan state laboratories. We do not contend that the salt virus is as virulent as blood virus, but that a salt solution does contain hog-cholera virus after remaining for a time in the peritoneal cavity of a virus hog. Virulence is no doubt acquired by the salt solution as a result of the phenomenon of osmosis, the interchange of substances occurring between the lymph spaces and blood vessels of all the abdominal viscera and the salt solution. It is possible that if a higher concentration of common salt were introduced into the peritoneal cavity, more virulence would be acquired by the salt solution.

The more economical production of antiserum warrants the use of salt virus and blood virus mixed in the intravenous method as well as the subcutaneous method. Assuming that the amount of blood secured from a virus pig is 10 to 15 c.c. per pound body weight, we find that the average pig used for production of virus yields approximately 200 to 500 c.c. more blood virus by volume when salt solution is

injected into the peritoneal cavity.   Including blood virus and salt virus, the volume of total virus solution is increased from 75 to 80%. The mixture of salt virus and blood virus must be used in larger doses in hyperimmunizing, but the difference between 5 c.c. per pound weight of blood virus and 7 c.c. per pound weight of blood and salt virus mixed does not offset the advantage gained.   In these different series, containing 3 or more immunes hyperimmunized by this method, the serum resulting proved to be protective when tested on susceptible pigs, and this fact is suggestive of the value of this method of producing antihog-cholera serum.

A FILTERABLE ORGANISM ISOLATED FROM THE TISSUES OF CHOLERA HOGS *

WITH PLATE 1

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Recently Healy and Smith[1] published a method whereby complement fixation may be obtained if a preparation of the mesenteric glands of the cholera hog be used as antigen. That this complement fixation is a specific reaction and not simply a reaction with the acetone insoluble residue, such as occurs in Wassermann's method, is strongly indicated in the work here reported.

Suitable mesenteric glands were selected from virus hogs, the glands carefully dissected out of mesentery, covered with 10 times their weight of absolute alcohol, and placed at 37 C. over night. The alcohol was then decanted off, the glands thoroughly ground with sterile sand, and the alcohol again added to the ground glands. The preparation was then placed at 37 C. for 8 days and thoroughly shaken each day. At the end of this period the preparation was filtered through an ordinary, white, folded filter paper. The filtrate, which measured 670 c.c., was evaporated by the aid of an electric fan to a somewhat pasty, deeply yellow mass. This mass was partly dissolved in 225 c.c. of ether and set aside to sediment. The ether was decanted from the sediment and allowed to evaporate to a volume of 15 c.c. To this 15 c.c. were added 75 c.c. (5 times its volume) of acetone. A heavy, flocculent precipitate immediately formed, which was allowed to settle, whereupon the supernatant liquid was decanted. Twenty-five cubic centimeters of methylic alcohol were added to the precipitate. The methylic alcohol dissolved a portion of the precipitate, leaving, however, a deeply yellow, sticky mass undissolved. After sedimentation the methylic alcohol, decanted and diluted 1 : 10 with normal salt solution (1.5 c.c.), was used as antigen in amounts varying from 0.01 c.c. to 0.03 c.c. with, in each case, 0.05 c.c. immune serum, 0.045 c.c. complement, 0.1 c.c. hemolysin, and 0.5 c.c. red blood corpuscles. The antigen, immune serum, and complement were mixed and placed at 37 C. for 1 hour, whereupon the hemolysin and corpuscles were added and the whole placed at 37 C. for 2 hours. Complete hemolysis followed in every case.

The test for complement absorption was negative. This test was repeated with 10 times the dose of antigen used in the first test, and the result was again negative.

In the light of these results it appears probable that our original antigen is specific. It is of interest that our original preparation of the mesenteric glands must remain for at least 8 days at a temperature

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[1] Jour. Infect. Dis., 1915, 17, p. 213.

of 4 C. for the antigen to develop, and it is of further interest that passage through an imported "F" Chamberland-Pasteur filter will remove the antigen from the preparation.

This preparation of mesenteric glands had been originally made in an effort to isolate a filterable organism from these glands. During the past two years we have made many such preparations and have endeavored to obtain a growth from them under a great variety of conditions—always, however, without success. On reviewing this work we noted that in the many attempts to obtain a growth, we had always filtered the preparation through an imported "F" Chamberland-Pasteur filter immediately after grinding the glands with sand. It therefore occurred to one of us (Healy) that a filterable organism might be present in such small numbers immediately after grinding and extraction of the glands that it could be readily filtered out by passage through an "F" bougie, but that if such a preparation was first placed at 4 C. for a period sufficient to allow some growth of the organism, and yet not long enough to exhaust its growth, and then filtered, it might be possible to pass an organism through the filter. This we succeeded in doing.

Mesenteric glands were obtained from 5 hogs which had been killed for the virus of acute cholera. The glands were carefully dissected from the mesentery and thoroughly ground with sand in an aseptic manner. To the ground glands was then added ten times their weight of a 1% glucose neutral beef broth, and the whole placed at 4 C. for 5 days. During this period an active fermentation occurred in this preparation. At the end of this period 100 c.c. of the supernatant liquid were passed through a reburnt, tested and sterile, imported "F" Chamberland-Pasteur bougie. The filtration required 40 minutes. The filtrate, which was brilliantly clear, was divided between 2 small, sterile Erlenmeyer flasks. One flask was placed in the Novy jar and the oxygen exhausted. The other flask was sealed with paraffin and placed in the air. Both flasks were placed at 4 C.

At the end of 4 days the flask placed in the air at 4 C. showed a distinct growth, which continued for about 2 weeks. This growth appeared as a fine sediment in the bottom of the flask. On the flask's being agitated with a circular motion, this sediment ascended through the fluid in the shape of a small cloud, resembling a delicate whiff of smoke. This is a characteristic appearance of cultures of this organism in fluid media. At the end of 13 days the flask placed in the Novy jar at 4 C. showed no evidence of growth. This flask was now sealed with paraffin and placed in the air at 4 C.; at the end of 7 days it

showed good growth, with the characteristic "whiff-of-smoke" appearance. This experiment was repeated 3 times, fresh virus glands being used for each experiment, and in each experiment we succeeded in obtaining the characteristic growth.

We demonstrated further that this filterable organism grows best at 37 C. in this extract of mesenteric glands, to which 5% sterile glycerin has been added. At this temperature the growth is comparatively rapid, and may be apparent at the end of 24 hours, reaching its maximal development in about 3 days. The organism also grows at 20 C. and 4 C., altho at these temperatures the growth is much slower and reaches its maximal development in about 2 weeks. The organism will not grow in the absence of oxygen, nor will it grow in any of the ordinary laboratory media, nor have we been able to obtain subcultures.

When the preparation of mesenteric glands is first made its reaction is very slightly acid. On standing at 4 C. this acidity increases in such manner that at the end of 5 days 100 c.c. of the preparation require about 4 c.c. of a N/N NaOH solution to neutralize it. It is of interest to note that the filterable organism will not grow in such a neutralized preparation.

A hanging drop preparation of this organism shows that it is non-motile, and that it occurs in very small clumps, showing many minute, bright points. In such a preparation the individual organisms cannot be clearly distinguished. It appears that the individual organisms occur in small clumps and are surrounded by gelatinous material, so that great difficulties are encountered when one attempts to stain them. With the ordinary preparations of the aniline dyes the gelatinous material either stains deeply throughout or does not stain at all, and the individual organisms are not apparent. Under the proper conditions Giemsa's solution affords an entirely satisfactory stain.

A 3-day-old culture at 37 C. was centrifugated and the precipitate smeared on thoroughly cleaned microscopic slides and air-dried. The preparations were then fixed by inverting them in methylic alcohol for 1 hour, rinsed in distilled water, and air-dried. They were then stained by inverting them for 1 hour in a warm preparation of Giemsa's solution containing 50 drops of the stock solution in 50 c.c. of distilled water, exactly neutralized according to the method given by Mallory and Wright.[2] This solution was prepared immediately before using and was warmed on the water bath for 5 minutes. After staining, the preparations were washed in distilled water, air-dried, and mounted in xylol balsam.

[2] Pathological Technique, 1911, p. 428.

In such a preparation the organisms are clearly and distinctly visible as minute bodies, stained deep-lilac in color, the gelatinous material being pale-lilac in color. The organism appears as a coccus, or a very small bacillus, of about 0.2 to 0.3 micron in diameter. Figure 1 is a retouched photomicrograph of such a preparation.

In our original paper we demonstrated that the antigen is removed from the 1% glucose broth extract of mesenteric glands by passing the extract through an imported "F" Chamberland-Pasteur bougie.

In addition to cultivating this filterable organism and staining it in a satisfactory manner, we have succeeded in fixing complement with cultures of the organism.

A glycerin (5%) glucose (1%) beef broth culture which had grown at 37 C. for 11 days was centrifugated and the clear supernatant liquid used as antigen (Table 1). The test for complement absorption was negative.

TABLE 1

Results of Complement-fixation Tests Using the Culture Fluid of the Filterable Organism as Antigen

Antigen in c.c.	Normal Salt Solution in c.c.	Immune Serum in c.c.	Complement in c.c.	Hemolysin in c.c.	Red Blood Corpuscles in c.c.	Hemolysis
.10	1.5	.05	.045	.10	.5	Complete
.13	1.5	.05	.045	.10	.5	Nearly complete
.15	1.5	.05	.045	.10	.5	Nearly complete
.17	1.5	.05	.045	.10	.5	Partial
.20	1.5	.05	.045	.10	.5	Very slight
.25	1.5	.05	.045	.10	.5	Complete
.30	1.5	.05	.045	.10	.5	Complete
	1.5	.20	.045	.10	.5	Complete

The organisms precipitated from this culture were suspended in 3 c.c. of normal salt solution and used as antigen in quantities of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, and 0.08 c.c., but with negative results. In addition to this the supernatant liquid was again passed through an "F" Chamberland-Pasteur bougie and used as antigen, with negative results.

SUMMARY

From the work it is evident that we have obtained an organism from the mesenteric glands of hogs acutely ill with hog-cholera; that we have passed this organism through an imported "F" Chamberland-Pasteur bougie; that we have successfully cultivated and stained this organism; and that we have obtained complement fixation with the culture fluid in which this organism has grown, such fluid not being able to fix the complement previous to the growth of this organism in it. These studies are being continued.

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EXPLANATION OF PLATE 1

Fig. 1. The filterable organism isolated from the tissues of cholera hogs.
X 1080.   Negative has been retouched.

Plate 1

Figure 1

THE MODE OF INFECTION IN PULMONARY DISTOMIASIS *

CERTAIN FRESH-WATER CRABS AS INTERMEDIATE HOSTS OF PARAGONIMUS WESTERMANII

PLATES 2 TO 5

KOAN NAKAGAWA
Formosa, Japan

Paragonimus westermanii Kerbert, the distome of the human lung is rather widely spread in the Far East. Ringer was the first to discover it, in 1879, in the lungs of inhabitants of the city Tamsui in Formosa. Since then it has been found in the various parts of Japan proper. In some places the distomes affect so large a number of the inhabitants that pulmonary distomiasis assumes almost an endemic nature. Consequently, the distome has been very carefully studied by various Japanese investigators. Altho it is now over 30 years since the discovery of the distome, the developmental stages of the worm, except its miracidia, have not as yet been clearly understood. For 10 years I have been carrying on an investigation[1] of pulmonary distomiasis in the locality of Formosa, where the disease is most prevalent, and finally

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[1] On the human lung distomes in the region Shinchiku, Jour. Taiwan Med. Assn., No. 138. Preliminary report on the discovery of the intermediate host of the human lung distomes, Tokyo-iji-shinshi, No. 1910. Study on the development of human lung distomes, Chugai-iji-shimpo, No. 1843; No. 1845.

I have been fortunate enough to discover the second intermediate host of the lung distomes. Recently the mode of infection has been experimentally determined.

THE DISTRIBUTION OF PULMONARY DISTOMIASIS IN FORMOSA

Before entering into details it seems proper to record here briefly the distribution of the cases of pulmonary distomiasis in Formosa; for it will show how I came to the discovery presently to be described.

Pulmonary distomiasis in Formosa was reported by Ringer in 1879. Nothing as to the distribution of cases was known until Nagayo reported in 1910 that cases were found in the northern region around Shinchiku. In the course of 1913-1914, I made a careful study of the disease throughout the whole island of Formosa, and found that of 1,249 patients 922, or about 73%, were residents of the Shinchiku Prefecture. This clearly shows that this prefecture is the center of infection (Map A). The distribution of patients in the central region of infection has been made clear by Matsuo, Yokokawa,[2] and others. They all agree that about 10% of the inhabitants of Shinchiku are affected by this disease. My microscopic examination of the expectorations of the boys and girls of the public schools revealed that 4.3% of all pupils were infected. This percentage means that not less than 1,300 cases must be present in Shinchiku Prefecture alone; the percentage may be somewhat larger for the whole population.

Altho nothing is known as to how the matter stands among the aborigines of Formosa, my frequent visits to their villages in 1914, convinced me of the fact that the nearer the valley they dwell, the more they are affected by distomiasis. Indeed, 50% of the inhabitants of such places were found to be infected. Among the people of the mountainous regions, the cases seem to occur far less frequently, tho my examinations were not numerous enough to warrant any definite conclusion.

INVESTIGATION OF THE SECOND INTERMEDIATE HOST OF PARAGONIMUS WESTERMANII

Appointed head of the Public Hospital at Shinchiku in 1912, I was placed thus under favorable conditions for carrying out an investigation of the lung distome. My attention was first directed to the search after the second intermediate host of the distome so that I might trace its developmental cycle. When I started the investigation, all that was known of the life-history was contained in Nakahama's and Manson's observations, that the miracidia hatched from the eggs begin to swim in about 4 weeks from the time of entering the water after they come out of the patients. As to what becomes of them afterwards, nobody had any idea.

[2] On the transmission path of the human lung distomes in the final host, Tokyo-iji-shimpo, No. 1910.

about 1000

about 200

about 50

about 10

under 5

Taihoku
189

Toyen
38

Giran
9

Shinchiku
922

Taichu

68

Nanto
5

Kagi
2

Region of Aborigines

Karenko
2

Pescadores Island

Tainan

Akaw
12

2

Taito

1
1.000.000

Map A. The distribution of patients with pulmonary distomiasis in Formosa. The map shows the number of patients in each prefecture during the years 1913-1914.

Collecting as many fresh-water molluscs as possible from the streams or ponds in the region where distomiasis exists as an endemic disease, I made careful search after the cercariae. I succeeded in obtaining 17 different kinds, but I could not distinguish the cercariae of the lung distomes from others. So I put various molluscs into the water in which miracidia of the lung distome were kept, to see what kinds of molluscs the miracidia would infest. It resulted that they infested Melania libertina Gould and M. obliquegranosa Smith most abundantly. From this it may be assumed that these two species of fresh water molluscs are the first intermediate hosts of the lung distomes. Notwithstanding the utmost care taken in keeping the molluscs infested with miracidia alive in an experimental pond, all of them died within a few weeks. They were found to contain no grown cercariae. Altho this experiment turned out to be a failure, I found that a certain kind of cercaria is found in all M. libertina living in the rivulets and creeks of the mountainous regions where the aborigines are infected in an enormous percentage.

The cercaria has a tadpole-like appearance, the body being 0.12 mm. long and 0.09 mm. wide, with a tail 0.054 mm. long. Attached to the oral sucker (0.036×0.032 mm.) are 2 pear-shaped bodies, the apices of which point towards the median plane of the body. The sucker has spines, each provided with a ring along its anterior edge. The ventral sucker is much smaller than the oral one, being 0.018 mm. in diameter. Within the parenchyma are 3 pairs of poison glands. The excretory vesicle is heart-shaped. Besides this kind of cercaria, the liver of the molluscs contains a good many sporocysts. Some melaniae have a number of half-grown cercariae. From the fact that M. libertina is found abundantly in the region where distomiasis of the lung prevails most widely, it may not be unreasonable to conclude that these cercariae are those of the lung distome. However, we have not as yet any experimental proof.

ENCYSTED LARVAE OF THE DISTOME OF THE HUMAN LUNG IN CRABS

Kobayashi's[3] interesting work on the discovery of the encysted larvae of Chlonorchis sinensis Cobbold in certain fishes gave me an impulse in the study of the question of the second intermediate host of the distome of the human lung. At first I selected a region of the Shinchiku prefecture where patients are abundant and made several difficult excursions to collect all molecules, fishes, amphibians, and

[3] A preliminary report on the second intermediate host and encysted larvae of the human lung distomes, Tokyo-iji-shinshi, No. 1918.

insects that I could. My microscopic examination showed that M. libertina Gould only has a certain kind of cercaria. In September, 1914, I captured in the rivulet in Kalapai Village a kind of crab which had not been caught before. My microscopic examinations were rewarded with the discovery of numerous encysted larvae in the liver. (Plate 3, Fig. 7; Plate 4, Fig. 8). They were all half grown, but they were unmistakably larvae of certain trematodes. At first I had no idea that they were the young distomes of the human lung. However, as the result of further investigation I found in the gills full-grown ones with all the morphologic structures peculiar to the distome of the human lung. Furthermore, I observed that the encysted larvae, when they were introduced into certain animals by mouth, grew into Paragonimus westermanii.

The young encysted larvae infest chiefly the liver of the crab, while the farther advanced ones are found in the gill. Sometimes they penetrate the muscle. The young encysted larvae are round, 0.2 mm. in diameter (Plate 2, Fig. 4). The young distomes in the cysts lie straight. They have a conspicuous, large, black excretory vesicle and comparatively large oral (provided with a spine) and ventral suckers. They do not have a distinctly developed alimentary canal. The encysted larvae found in the gill are well developed, measuring 0.3 to 0.4 mm. in diameter (Plate 2, Fig. 5). The young distome in the cyst has a short and thick body and lies straight, unlike others, which are contorted. The oral sucker has a spine, and is 0.08 to 0.11 mm. in diameter. The short esophagus is connected with the bifurcate intestine. Each branch of the intestine is thick and undulating, running parallel to, and outside of, a long, thick excretory vesicle. The ventral sucker is a little larger than the oral one, measuring 0.07 to 0.12 mm. in diameter. The entire surface of the body is provided with short spines. The wall of the cyst is 0.01 mm. thick—a characteristic feature of the species. The young distome rotates sluggishly in the cyst. The full-grown encysted larvae sometimes reach 0.5 mm. in diameter (Plate 2, Fig. 6), and to the naked eye look like a white speck. Sometimes they assume an elliptical shape, 1.0 mm. long and 0.4 mm. wide. They can readily be removed from the gill. Twenty percent of larvae thus freed were observed to float on the surface of the water. Under natural conditions the full-grown larvae would leave the crab's gills, get into the water, and be taken up by the dwellers on the banks of the lower stream.

Map B. Showing the correlation between cases of pulmonary distomiasis and infested crabs.

FRESH-WATER CRABS WITH THE LARVAE OF PARAGONIMUS WESTERMANII

Potamon obtusipes (*Stimpson*)—"Red crab" (Plate 2, Fig. 1). This was the first of the three species of crabs in which the encysted larvae of the human-lung distome were found. The carapace is coarse, flat, and provided with teeth along the lateral margins; the pterygostomian region is granular; the dorsal surface is a deep-chestnut color; the abdomen and legs are somewhat reddish. One large specimen has a carapace over 38 mm. in diameter. The native name of this crab is "shahoi" or "chahoi," which means red crab. Sometimes it is eaten. This species has never been found in any place in Japan other than the mountainous region in Formosa. I have found that the crab is most abundant in rivulets or creeks running through the mountainous regions of Shinchiku Prefecture. The number of encysted larvae in the gills increases in correlation with the number of cases of distomiasis of the lungs; for instance, in the region where 30-50% of the inhabitants are found to harbor the parasites in their lungs, 100% of the crabs carry the encysted larvae, while 5 miles down the river, where comparatively few patients are met with (I have had no opportunity to make sure of the percentage), only 11% of the crabs have the encysted larvae. In the crabs of Sansaka region no encysted larvae were found (Map B).

Potamon dehaanii (*White*).—This species (Plate 2, Fig. 3) occurs in the same locality with the species mentioned, but not so abundantly, and it is a little smaller in size. The carapace is somewhat round and smooth. There are no teeth on the lateral margin. The pterygostomian region is smooth. The dorsal surface is grayish-black, or sometimes slightly reddish. The ventral side and the legs are grayish-white. This species also occurs in the rivers of the mountainous regions in Japan proper. The natives, who call it "sai-hoi" (dung crab), do not eat it. Encysted larvae of the distome of the lung were also found in this species, tho less numerously than in the former.

Eriocheir japonicus De Haan.—Unlike the other two species, this one (Plate 2, Fig. 2) never occurs in the streams of the mountainous region, but occurs in the rivers flowing across the plain. This species can easily be discriminated from the other two by its chelae, which are provided with a thick growth of hair. Very big specimens have a carapace over 76 mm. in diameter. The native name is "mon-hai," or "mun-hai" (hairy crab). This crab is eaten by the natives. The

er-cysted larvae were found in only 2 out of 330 specimens (300 large
and 30 smaller ones) which I collected and examined. I am not certain
whether or not the attachment to the crabs is merely an accident. The
solution of the problem awaits further investigation.

In conclusion I may say that of the three species of fresh-water
crabs the encysted larvae of the human lung distomes are found in
the first and the second species, the occurrence in the third being prob-
lematical. In view of the fact that the second species occurs outside
Formosa, this may be the second intermediate host of distomiasis in
Japan proper.

EXPERIMENTAL PULMONARY DISTOMIASIS

In order to verify experimentally the conclusion reached in the
last section, I secured some dogs that had been brought up in a place
where no cases of distomiasis of the human lung are known. I used
dogs because they are known to be easily infected by the lung distomes.
The liver, the gills, and other organs of the crab that harbored a large
number of encysted larvae were given to 2 puppies on September 23
and October 10, 1914. One of the dogs died December 9; that is, 60
days after feeding. Postmortem examination showed the lungs to
have a number of cysts. Within each cyst 2 or 3 distomes were
present. They had no eggs in the uterus at this time. They
measured 4 to 5 mm. in length and 2 to 3 mm. in width. The other
puppy died on December 27 after a lapse of 90 days from feeding.
His lungs contained numerous cysts, in which adult distomes with
eggs ready to be discharged were found. These distomes measured
6 to 7 mm. in length and 3 to 4 mm. in width—not larger than half the
ordinary size of the distomes found in the lungs of man, the cat, and
the dog. Their morphologic features, however, tallied well with those
of Paragonimus westermanii. The worms thus raised in the lung of a
puppy have a short, thick body, dark in color in the living stage. They
assume various irregular shapes while in physiologic salt solution.
After being fixed in formalin they are grayish-white and ovoid, the
anterior end being rounder than the posterior. The transverse section
is nearly circular.

The experiment was repeated at the Shinchiku Public Hospital
with 3 other puppies that had been brought from a region free from
lung distomiasis. Two were fed with a large quantity of the internal
organs of crabs, while the remaining one was left untreated as control.

The two animals died 50 days after feeding; that is, on February 7, 1915. A number of distomes were found in their lungs. The control animal, however, was found to be free from them when I examined its lungs on the same day. Again, another puppy, to which water containing some encysted larvae was given, contracted the disease.

MODE OF TRANSMISSION OF THE DISTOME OF THE LUNG IN THE FINAL HOST

In order to ascertain how the distome finds its way into the final host, and to see what pathologic changes the lungs undergo, I made examination of puppies at various intervals after infection.

On the fifth day from feeding no macroscopic changes were visible in the lung. On the seventh day 2 or 3 fresh petechiae, not larger than a pinhead, were seen. On the fourteenth or fifteenth day hemorrhage was obvious on the surface of the lung, especially on the lower lobes. From the twenty-first to the twenty-seventh day small nodules made their appearance, besides hemorrhagic spots. Generally the nodules were in connection with the hemorrhagic spots, but sometimes the former appeared quite independent of the latter. On the thirty-fifth day, hemorrhagic spots as big as linseeds were visible. On the fiftieth day, large nodules or cysts of Paragonimus westermanii appeared, which were as big as the end of the little finger. Some of the nodules bulged out a little on the surface of the lungs. They were rather hard and of a dark-red and gray color. The section was of a dark-red color, which might have been due to the hemorrhage. In the center of such nodules the distomes were embedded. Sometimes the worm lay embedded in surrounding tissues. On the ninetieth day, the cysts sometimes assumed a bluish-gray color. On section they were found to have a cavity as large as a pea, filled with a porridge-like substance in which one or two fully grown worms were present. The walls of such cysts were formed of a thin layer of connective tissue.

Two puppies were fed with grown encysted larvae from April 15 to April 27, 1915 (both died of emaciation); likewise 3 kittens—No. 1 from April 18 to May 2; No. 2 from April 18 to May 16; No. 3 from April 25 to May 16. I chloroformed these animals and took out all the internal organs. The abdominal cavities were filled with comparatively large quantities of serous secretion. The livers were generally congested. The diaphragms had numerous small perforations

like passages made by small distomes. Over the surface of the liver and the omentum major in each animal were seen many distomes attached. On the mucous membrane of the jejunum and spleen one petechia, or two, or sometimes more, was seen of the size of a pinhead or sometimes larger. Hemorrhagic spots were present on the pleura; on the thoracic side of the diaphragm were minute petechiae and small perforations; the lung had many hemorrhagic spots and small nodules.

On examination of microscopic sections through a petechia of the jejunum, the worms were found to have penetrated through the mucous membrane and inner muscle layer, and to be about to bore through the external layer (Plate 4, Fig. 9). It is obvious that these worms had come out of the cysts and penetrated the intestinal wall. Moreover, the passage through the muscle layer was indicated by the interruption of the muscle layer and by a marked infiltration of leukocytes, especially of eosinophile cells. Preparations of omentum major showed that it had some worms embedded in the net-tissue and also in the fat tissue near the region (Plate 5, Fig. 10) where it is united with the large curvature. In the sections of the petechia of the diaphragm worms were found just penetrating the tendon (Plate 5, Fig. 11). The penetration path had the same microscopic features as that in the intestine. In the petechiae of the spleen and the kidney no worms were seen. In the sections of the lung of a puppy killed 30 days after being fed with encysted larvae, were numerous hemorrhagic spots, but no worms were found embedded in the tissue.

The distomes that had reached the thoracic cavity had not as yet penetrated the lung. It may be conjectured that they live for some time floating in the serous excretion of the thoracic cavity or under the pneumopleura, and attached by suckers to the lung-parenchyma, thus causing small petechiae there. Of the worms in the thoracic cavity only a small percentage seemed to get into the lung; for the number of such nodules was far less than that of the worms in the serous liquid. The sections of the nodules showed a trace of loss of tissue and a high degree of infiltration of leukocytes (especially eosinophile cells) around it. This was undoubtedly the path through which the worms had penetrated. But the worms had not developed into cysts there; in more than 50 days from the infestation, the cysts would have been completely formed.

The wall of a newly formed cyst consisted of young connective tissue with an infiltration of great numbers of polymorphonuclear

leukocytes and small cells, while that of an old one was formed of a strong, fibrous connective tissue. The wall, however, did not present a uniform structure; in some places the cyst was completely lacking in walls, being directly apposed to the alveolar tissue of the lung, the small bronchi, or veins, thus causing local bleeding and pneumonic infiltration of the alveoli. Within each cyst lay one or two worms. Sometimes one or two of the worms that lay in the same cyst, would be found decomposing. The eggs were observed to have been laid first on the ninetieth day after feeding. They lay in the cyst or the parenchyma of the lung around the cyst, and generally were found mixed with erythrocytes, leukocytes, epithelial cells, and their products. Near the pathologic regions dilatation of bronchi was present, the cavity being filled with erythrocytes, eggs, etc.

From what has been described it may be seen that the lung distome will form a cyst in the lung of the host, live only for a while in it, and then leave it, finding its way through the tissue, bronchi, blood-vessels or the wall of the cyst by virtue of a vigorous motion that causes local bleeding and pneumonic infiltration. The degenerative products will be discharged from the lung through the trachea, carrying eggs with them.

To sum up, the encysted larvae of Paragonimus westermanii that have been taken into the alimentary canal of the host, creep out of the cysts and making their way through the intestinal wall near the jejunum, reach the abdominal cavity. They then penetrate the diaphragm and reaching the thoracic cavity, scatter all over the space under the pleura, whence they can easily find their way to the lung. They pierce the lung parenchyma, and there the cysts are formed until they become fully grown. These parasites can bore through various tissues and may reach other organs than the lung, where they form their regular cysts; but the lung seems to be the most favorable place for their development and the laying of their eggs; in other organs they can never reach the perfect growth.

EXPLANATION OF PLATES

Plate 2.   Fresh-water Crab

Fig. 1. Potamon obtusipes (Stimpson) nov. sp., natural size.

Fig. 2. Eriocheir japonicus De Haan nov. sp., natural size.

Fig. 3. Potamon dehaanii (White), natural size.

Fig. 4. Young encysted larva in the liver of a crab (Potamon obtusipes). × 20.

Fig. 5. Half-grown encysted larva in the liver of a crab. × 20.

Fig. 6. Full-grown encysted larva on the gill of a crab. × 20.

Plate 3

Fig. 7. Encysted larvae of Paragonimus westermanii in the liver of a fresh-water crab (Potamon obtusipes).

Fig. 8. Encysted larvae of Paragonimus westermanii in the liver of a crab. × 15.

Plate 4

Fig. 9. Cross-section of the jejunum of an experimentally infected kitten (No. 1). Young distome is seen passing through the intestinal wall. × 20.

Fig. 10. Adipose tissue of the omentum of an experimentally infected kitten (No. 1). a = worm. × 50.

Plate 5

Fig. 11. The diaphragm of an experimentally infected kitten (No. 1). a = young worm in the tendinous part of the diaphragm. × 50.

Fig. 12. Section through the lung of an experimentally infected puppy (No. 1) 60 days after feeding. a = passage of the worm. × 20.

PLATE 2

fig.1

fig 2

fig 3
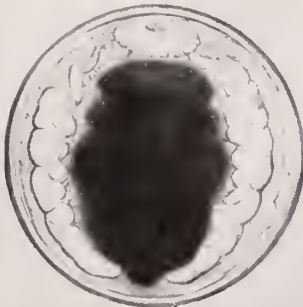
Fig. 4

Fig. 6

Fig 5
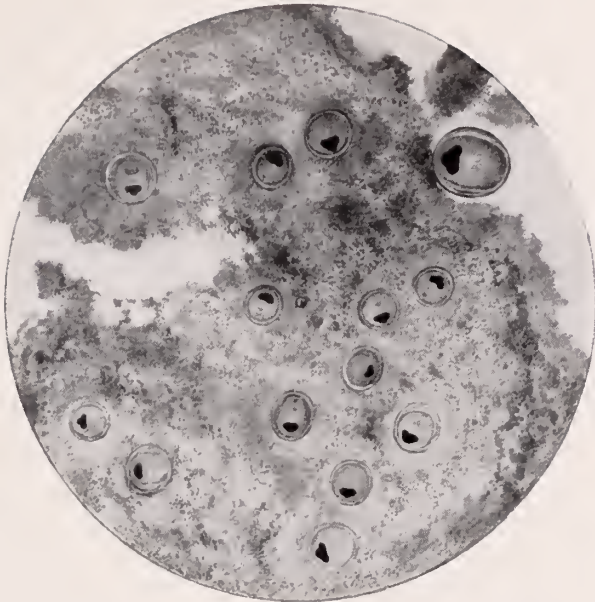
PLATE 3

Fig. 7
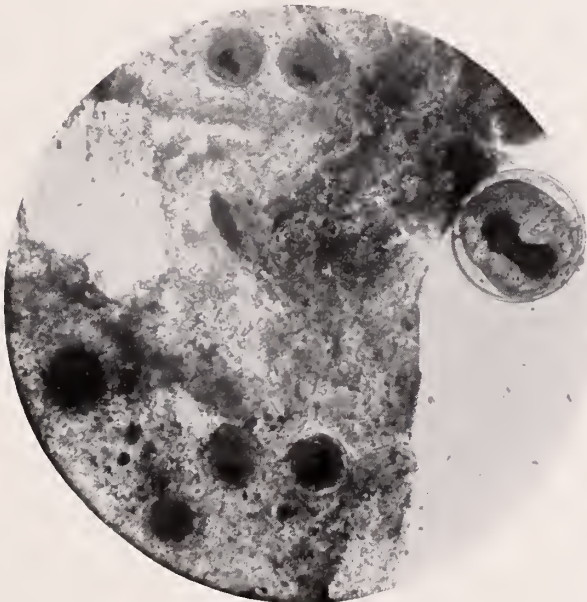
Fig. 8

PLATE 4
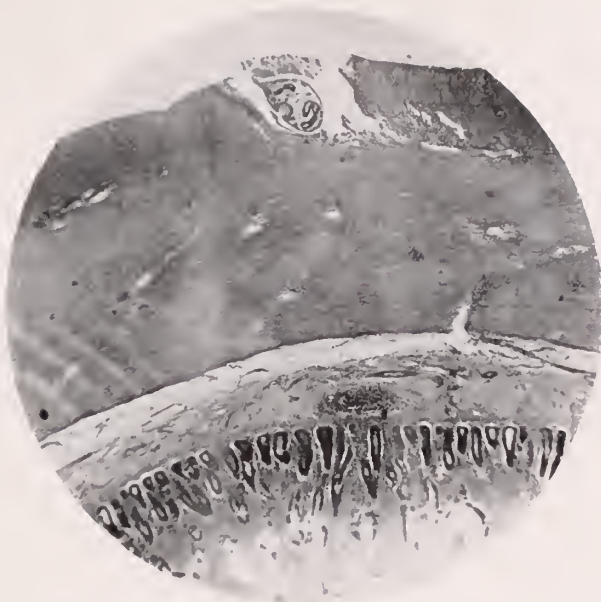
Fig. 9
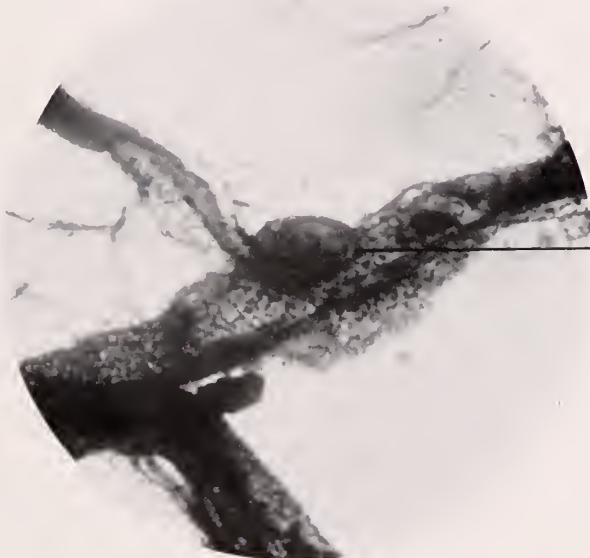
Fig. 10

Plate 5

Fig. 11

Fig. 12

A MILK-BORNE PARATYPHOID OUTBREAK IN
AMES, IOWA *

MAX LEVINE AND FREDERICK EBERSON

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The frequency of paratyphoid fever in man is an unsettled question. Clinically the disease simulates true typhoid fever so closely that a diagnosis of the latter is almost always made; nor can the routine agglutination test with typhoid bacilli be relied upon to differentiate between the two diseases. Accurate statistics on the prevalence of paratyphoid infections are therefore lacking.

Conradi[1] found 18 paratyphoid cases among 235 patients apparently ill with typhoid, and Brion and Kayser,[2] in an investigation of 300 cases of fever during the two years, 1904-06, found 9 cases of paratyphoid infection. Seven of the latter were caused by B. paratyphosus B and 2 by B. paratyphosus A. In a study of 505 cases in Strassburg, Kayser[3] found 27 of the paratyphoid B, and 5 of the paratyphoid A type. According to Brown and Deeks,[4] 50% of apparent typhoid fever in the Canal Zone is really paratyphoid fever. .

Literature on the epidemiology of paratyphoid fever is meager. Infected meat is generally regarded as an important means of spreading the disease. Several small outbreaks due to water and milk have been reported abroad. Fischer[5] reports 50 cases of paratyphoid which he attributes to a milk supply infected through 2 cows suffering from enteritis. In another outbreak, recorded by Nielsen,[6] the milk supply had been infected by a girl who nursed 2 paratyphoid patients in a neighboring town. In Graesvick, Germany, Gram[7] traced 16 cases to a milk supply and isolated the paratyphoid organism from river water which was used in the cow barn.

The outbreak which we are about to describe was small in extent, but since the causative organism was B. paratyphosus and milk the vehicle of infection we deem it of sufficient importance to be put on record.

THE PARATYPHOID OUTBREAK IN AMES

Ames is a typical college community with a population of 5000, exclusive of about 2700 students. It is situated on the main line of the Chicago and Northwestern railroad running east and west, and is the central point for several branch lines running north and south, as well as for the Interurban

[1] Klin. Jahrb., 1907, 17, p. 351. Deutsch. Med. Wchnschr., 1904, 30, p. 1165.
[2] München. med. Wchnschr., 1902, 49, p. 611. Arch. f. klin. Med., 1905, 85, p. 525.
[3] Centralbl. f. Bakteriol., I, O., 1912, 40, p. 285.
[4] Cited, Kolle and Wassermann, Handb. d. pathogen. Mikroorganismen., 1913, 3, p. 1012.
[5] Klin. Jahrb., 1906, 15, p. 61.
[6] Tidsskr. f. d. norske Laegefor., 1907, 27, p. 333.
[7] Centralbl. f. Bakteriol., R., 1910, 48, p. 171.

railway, which communicates with a large number of outlying municipalities throughout the state. The location of the city, coupled with its migratory population, makes it an important focus for the spread of an epidemic.

Typhoid fever is not a reportable disease in the state of Iowa. Consequently, altho cases had been present in the city of Ames for several months, the health authorities were not aware of the fact. The occurrence of several cases in the family of one of the members of the faculty brought the matter to the attention of the college authorities. On November 14, this laboratory was authorized by the state board of health to undertake a complete study of the outbreak.

A canvass among the physicians revealed that, during July and August, 4 cases had been reported, none in September and October, and 11 between November 1 and 16. The four cases first mentioned were apparently not interrelated. Two were traced to out-of-town sources; the sources of the other two could not be determined. One of these may have been the focus of infection in the November outbreak, and will be discussed more in detail. A table of the November cases follows:

TABLE 1

THE CASES OF PARATYPHOID FEVER DURING NOVEMBER

Case	Date of Taking to Bed	Number of Cases
1	November 1	1
2	November 4	1
3, 4	November 6	2
5	November 8	1
6, 7	November 9	2
8	November 10	1
9*	November 11	1*
10	November 12	1
11	November 16	1

* Infection came from out of town (Arkansas); it will not be included in this discussion.

Case 1 was that of an adult who spent considerable time traveling about the country. The infection was first diagnosed as pneumonia; agglutination tests with typhoid bacilli made on November 12 and 15 were negative. A test made on December 20 gave a marked positive reaction. It is very likely that this case was an out-of-town infection.

Cases 2, 3, and 4 gave a history of weakened resistance through continuous, or intermittent, illness throughout the summer. The date of first symptoms in these instances, therefore, could not be definitely determined.

Cases 10 and 11 were due to contact infection. The former was in a family in which 2 other cases (3, 4) occurred on November 6. The latter (11) was in intimate contact with Case 6 during the prodromal period, and for at least 2 days after definite onset. Both these patients were inoculated with typhoid vaccine several days before coming down.

From the foregoing considerations it seems apparent that the actual period of causation was extremely short, perhaps not more than a day or two in the proximity of October 25. There seems to have been a prodromal period of 4 or 5 days.

Possible Sources of Infection other than Milk.—A large majority of the patients obtained their fruits and raw vegetables from the same dealer, but relatively few gave a history of eating the same kinds of raw vegetables.

Furthermore, this dealer supplies a large part of the community throughout the city, so that if these raw products were responsible for the outbreak, the cases ought to have been scattered throughout the city. In this outbreak all the cases were in a small section of the town. A study of the distribution of the cases as to ice cream consumption eliminated this food as a probable cause. The season of the year served to eliminate flies and insects.

The city water supply, obtained from 2 well-constructed, deep wells, is of excellent quality bacteriologically. Nine patients used city water exclusively. The tenth obtained water from a private well, but had access to city water at school. The extreme localization of the cases coupled with the wide distribution of the public water supply removes suspicion from this source.

Milk, the Probable Source of Infection.—Of the 10 patients 9 obtained milk exclusively from one dealer "D" during the causation period and up to the time of this investigation. The tenth had some milk from this dealer, but the infection was probably due to contact. Seven gave a history of drinking raw milk habitually, 2 had very little milk, and 1 had milk only with cereals for breakfast.

The distribution of the cases with reference to age points to milk rather than to water or other food substances as the vehicle of infection. Seventy percent of all the cases were among children under 14 years of age; or, if we omit the two cases due to contact, 62.5% were among children of milk-drinking age.

TABLE 2

The Distribution of Cases of Paratyphoid Fever According to Age and Sex

Age	Male	Female	Total	Percentage
0- 4	1	1	2	20
5- 9	1	1	2	20
10-14	1	2	3	30
25-29	...	1	1	10
30-34	...	1	1	10
45-49	1	...	1	10
Totals	4	6	10	100

The infected milk supply was obtained from a dozen farms in the vicinity of Ames. Careful investigation of these sources failed to disclose any typhoid-like case, and all evidence as to visitors and convalescents on these farms, as well as disease among the herds, was negative.

There were 2 possibilities for bottle infection. A case of apparent typhoid fever came down on July 28. On September 1, milkman "D" began to deliver milk to this patient. The patient did not leave her bed until October 15. From this date she gradually resumed her household duties. Such precautions as disinfecting stools and urine were discontinued, and about 3 weeks after her apparent recovery, her daughter came down with the fever (Case 7, Nov. 9). The possibility of bottle infection by this convalescent is evident.

On the other hand, there was opportunity for infection of the milk supply at the dairy. The milk dealer stated that he had had typhoid fever 4 years previous and that his wife and 4 children had all been down with the disease at that time (1910). An agglutination test (microscopic) with typhoid bacilli gave a questionable reaction with serum from the milk dealer's wife; bacteriologic examination of her feces showed the presence of paratyphoid-like organisms. This woman had charge of the washing and filling of the bottles, which were not sterilized, but merely rinsed in cold water.

The occurrence of all the primary cases in a restricted area of the community and on one milk route, the high percentage of infection among children, and the location of 2 possible sources of infection of the milk supply, are conclusive for the outbreak's having been milk-borne.

BACTERIOLOGIC FINDINGS

The proximity of the laboratory made it possible to use freshly collected blood samples for the bacteriologic diagnosis, thus being eliminated the possible errors of dilution in dried specimens. For diagnosis about 0.25 c.c. of blood was collected in a Wright capillary pipet, allowed to clot, and centrifugated. In the course of the routine agglutination tests with typhoid bacilli it was observed that agglutination of B. typhosus with a 1 : 40 dilution of the centrifugated serum was extremely slow (usually absent for an hour or more), while one specimen (Case 7) which was tested simultaneously against B. paratyphosus B brought about complete clumping of this organism in less than 15 minutes. Attention was therefore directed to the possible presence of some organism other than B. typhosus, and to the possibility that the outbreak was one of paratyphoid fever rather than true typhoid. Samples of urine and feces from 3 carrier suspects and 8 patients were examined for the presence of the causative organism, and macroscopic agglutination tests were made with B. typhosus and the A and B types of B. paratyphosus.

Isolation of Organisms.—The technic finally employed for the isolation of the causative organisms was as follows: Samples of feces and centrifugated urine were smeared directly onto Conradi-Drigalski plates; at the same time lactose bile tubes were inoculated with urine and feces, respectively, and after 4 to 5 hours' incubation at 37 C. new Conradi-Drigalski plates were made. All plates were incubated at the body temperature for 24 hours. If suspicious colonies developed, they were fished, streaked on fresh Conradi plates, and re-incubated. From this second series of plates several suspicious colonies (10 or 12) were fished onto agar. The cultures thus obtained were then inoculated into a series of sugars and various other confirmatory tests, to be described, were made.

In no instance did we obtain B. typhosus, while organisms of the paratyphosus type were secured in pure culture from the feces of a carrier suspect (the milk dealer's wife) and from the urine of 1 of the patients. On the Conradi plates, from the feces of 2 other patients, bluish-gray colonies were present which were identical in appearance

with those obtained from carrier and urine, but we did not succeed in separating them from B. coli. The cultural and morphologic characters of the isolated organisms are given in Table 3.

TABLE 3

Cultural and Morphologic Characters of Organisms Obtained from Paratyphoid Patients

Organism	M_2	R_1	R_3
Source	Feces of carrier suspect	Urine of Patient 8	Urine of Patient 8
Conradi-Drigalski plates	Colonies small, of a bluish-gray color with medium discolored slightly bluish		
Gram stain	—	—	—
Motility	+	+	+
Morphology	Short rod	Short rod	Short rod
Dextrose	+	+	+
Lactose	—	—	—
Saccharose	—	—	—
Maltose	±	±	±
Levulose	+	+	+
Galactose	+	+	+
Mannite	+	+	—
Raffinose	—	—	—
NO₃ reduced	+	+	+
Indol	+	—	+
Milk	Acid then alkaline	Acid	No change

From Table 3 it is evident that the isolated organisms belong to the paratyphoid group. It will be observed that indol was produced by 2 of the strains. The power to produce indol is not generally ascribed to B. paratyphosus, but Poppe[8] found that indol-production by B. para-typhosus was dependent upon the grade of peptone employed in the test, while Andrejew working with a series of strains of B. para-typhosus B repeatedly obtained positive indol reactions. One of us[9] has observed indol-production in paratyphoid cultures isolated from cholera-infected hogs.

The cultural tests were confirmed by the use of differential media. The materials used were: Loeffler's malachite-green dextrose nutrose

[8] Ztschr. f. Infektionskrankh. d. Haustiere, 1908-09, 5, p. 42. Ztschr. f. Immunitätsf., O., 1912, 13, p. 185.

[9] Eberson: Jour. Infect. Dis., 1915, 17, p. 331.

solution (Loeffler I); Loeffler's malachite-green lactose nutrose solution (Loeffler II); Barsiekow's litmus nutrose dextrose solution (Barsiekow I); Barsiekow's litmus nutrose lactose solution (Barsiekow II); Hetsch's litmus nutrose mannite solution; Petruschky's litmus whey; plain milk, dextrose broth, lactose broth; and orcein agar and neutral-red agar. The results are shown in Table 4.

TABLE 4

CULTURAL RESULTS WITH USE OF DIFFERENTIAL MEDIA

Strain	Loeffler		Barsiekow		Hetsch	Litmus Whey	Milk	Dextrose	Lactose	Orcein Agar	Neutral-red Agar
	I	II	I	II							
M 2	Coagulated	Unchanged	Coagulated; acid	Unchanged	Coagulated; acid; slight gas	Alkaline	Unchanged	Gas	No gas	Cleared	Fluorescence; gas
R 4	Coagulated	Unchanged	Coagulated; acid	Unchanged	Coagulated; acid; slight gas	Slightly acid	Unchanged	Gas	No gas	Cleared	Fluorescence; gas
Paratyphosus B	Coagulated	Unchanged	Coagulated; acid	Unchanged	Coagulated; acid; slight gas	Alkaline	Unchanged	Gas	No gas	Cleared	Fluorescence; gas

Agglutination Tests.—A rabbit was immunized against M$_2$ by repeated intravenous injections of an agar culture, which was suspended in salt solution and heated at 60 C. for 30 minutes. Ten days after the last injection the animal was bled and the serum tested macroscopically against the homologous strain, and against strains of B. paratyphosus A and B. It was found that a 1:1200 dilution of the serum agglutinated the homologous strain, but that a 1:100 dilution would not agglutinate B. paratyphosus A and B. Another rabbit was immunized against B. paratyphosus B and the serum tested against the organisms isolated. The tests were negative.

From the agglutination tests it is apparent that the serum specific for B. paratyphosus B did not agglutinate the isolated strains and vice versa; but this lack of confirmation of the cultural characters does not vitiate the conclusion that the organisms are of the paratyphoid group. We may cite Messerschmidt,[10] who isolated from a paratyphoid fever patient an organism which simulated B. paratyphosus B in all cultural characters, but which was not agglutinated by sera produced by any member of the typhoid-colon group. Under certain conditions organisms which are well agglutinated may become non-agglutinable. According to Paltauf,[11] certain strains are really composite, possessing both agglutinable and non-agglutinable members,

[10] Centralbl. f. Bakteriol., I, O., 1912, 66, p. 35.
[11] Kolle and Wassermann, Handb. d. pathogen. Mikroorganismen., 1913, 2, p. 502.

and the latter may predominate at times.   The agglutination test is
rather variable ; many observers have recorded the isolation of strains
which were but slightly agglutinable, if at all.

The conclusion we have reached, that this outbreak was due to
the paratyphoid infection, was not based merely on the isolation of
suspicious organisms, but primarily on tests obtained with the sera of
the patients.

On December 20, about 6 to 7 weeks after the onset of the cases,
blood samples were collected from 4 patients and tested macroscopically
against B. typhosus, B. paratyphosus A, and B. paratyphosus B.   The
results are shown in Table 5.

TABLE 5

MACROSCOPIC AGGLUTINATION TESTS WITH TYPHOID BACILLI AND SERA FROM PATIENTS

Case	Organism	Dilution of Serum				
		1-100	1-200	1-500	1-800	1-1200
3	B. typhosus..............................	—	—	—	—	—
	B. paratyphosus A......................	+	—	—	—	—
	B. paratyphosus B......................	+	+	+	—	—
11	B. typhosus..............................	—	—	—	—	—
	B. paratyphosus A......................	+	—	—	—	—
	B. paratyphosus B......................	+	+	±	—	—
8	B. typhosus..............................	—	—	—	—	—
	B. paratyphosus A......................	+	±	—	—	—
	B. paratyphosus B......................	+	+	±	—	—
1	B. typhosus..............................	+	+	+	+	+
	B. paratyphosus A......................	—	—	—	—	—
	B. paratyphosus B......................	—	—	—	—	—
9 (a)	B. typhosus..............................	—	—	+	+	+
	B. paratyphosus A......................	+	—	—	—	—
	B. paratyphosus B......................	+	—	—	—	—

Case 9(a) was not connected with the outbreak.   This was a
typical typhoid patient, who became infected while in Arkansas.   The
serum was employed as a control.   Lack of agglutination in the low
dilutions is probably due to the presence of pro-agglutinoids.

From Table 5 it appears that Case 1 is markedly different from the
others.   The epidemiologic evidence was also of a nature to throw
doubt on the connection of this case with the outbreak.   The patient
was an adult about 50 years old, who spent considerable time traveling
about the country.   It is possible that infection in this instance occurred
out of town.

The other three sera agglutinated B. paratyphosus B in higher dilu-
tions than B. paratyphosus A, while B. typhosus was not clumped.   The

sera were not very potent. The agglutinins reach a maximum in about
2 weeks after which there is a rapid decrease. In view of the fact that
these tests were made about 6 to 7 weeks after the onset of the cases,
more potent sera could hardly be expected.

Case 3 was in a home where 2 others were affected, while Case 11
was contracted through contact with Case 6. Earlier in the investi-
gation one patient (7) agglutinated B. paratyphosus B much more
rapidly than B. typhosus. Thus, it is quite conclusive that at least 7
patients were affected with paratyphoid fever.

SUMMARY AND CONCLUSIONS

For the following reasons we feel satisfied, from a study of the
epidemiologic evidence, that milk was the vehicle of infection.

All the primary cases were on one milk route. Of the two cases
attributed to contact, one used this milk exclusively, the other occa-
sionally.

Sixty-two and five-tenths percent of the primary infection was
among children, all under 14 years of age. This high percentage of
infection is characteristic of milk-borne outbreaks.

There are 2 possible sources of infection of the suspected milk
supply.

The age incidence and the localization of the cases eliminate other
food supplies, such as water or vegetables.

From the bacteriologic study of the cases, it appears that after 6
to 7 weeks the patients' sera were more potent towards B. paratyphosus
B than towards the A type, while the typhoid organism was not
agglutinated. This, together with the atypical clinical course and diag-
nostic reactions of agglutination with typhoid bacilli leads us to regard
the outbreak as one of paratyphoid fever.

Altho it is not possible definitely to ascertain whether the infection
was from a carrier or a convalescent, there is no doubt that proper
handling and bottling of the milk would have forestalled this outbreak.
The two cases attributed to contact were both inoculated against
typhoid several days before coming down. This was before the disease
was recognized as paratyphoid fever. Immunization against para-
typhoid might have protected these patients. This outbreak points
out the necessity of testing questionable and negative typhoid speci-
mens against the paratyphoid strains.

THE VARIATIONS IN REACTION OF THE BLOOD OF DIFFERENT SPECIES AS INDICATED BY HEMOLYSIS OF THE RED BLOOD CELLS WHEN TREATED WITH ACIDS OR ALKALIES *

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In 1913, at the Pasteur Institute of the University of Michigan an attempt was made to establish the Wassermann reaction on a chemical basis. The research in this direction dealt chiefly with the influence of salts, acids, and alkalies on the hemolytic system. It was first noted that any one of these, even in high dilution, had its influence on this biologic reaction. A specimen known to be negative to the Wassermann test, could be made to give a positive reaction by the addition of a trace of either acid or alkali. Furthermore, on the addition of a somewhat larger quantity of acid or of alkali, a positive specimen would give a negative reaction. Similarly, neutral salts would influence a negative specimen to the extent of causing a positive reaction. The opposite of this, however, was not true.

Further experiments developed the fact that a series of positive and negative reactions, analogous in result to positive and negative Wassermann reactions, could be produced by an acid solution as antigen, and by rabbit serum, rendered acid or alkaline, as positive or negative serum. The acid serum would give a positive end result; while the alkaline serum would be negative. The analogy between the final results of these experiments and those of the Wassermann, coupled with the fact that the actual Wassermann could be influenced with acids or alkalies, and a negative specimen influenced with neutral salts, led to the assumption that the positive Wassermanns might be due to an increase in ions during the first incubation, either acid or alkaline ions, or those of neutral salts.

This assumed to be a fact, the next step in the work was an investigation of the action of acids and alkalies on the hemolysis of certain blood cells. By allowing a fixed time interval for complete hemolysis of a definite blood cell suspension, the percentage of acid or of alkali

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necessary to produce this result could be determined, and this determination could remain fixed for the species thus tested. If a positive Wassermann were due to an increase in ions, either acid or alkali, during the first incubation, the acid or alkaline hemolysis of the second incubation could be correspondingly influenced by either a retardation or an acceleration of the reaction. Should the increased ions be of an alkaline nature, and an acid be used as a hemolytic agent, the time interval would be prolonged; or, if the hemolysin should be alkaline, the interval would be shorter than that of the control. On the other hand, if the increased ions were acid and the hemolytic agents were used in the same order, the results would be reversed. That is, the acid ions produced during the first incubation, coupled with those added as the hemolytic agent, would hasten the reaction, and would retard alkaline hemolysis. At this time the investigation of the hemolytic action of acids and alkalies was limited to the blood cells of 4 species: man, goat, dog, and horse. There was a slight, but well-defined, difference between the blood suspension from man and that from the goat, and between that from the horse and that from the dog. The difference, however, between the blood suspensions of the first group and those of the second was extremely marked,—relatively large quantities of chemical hemolysin being required for the first group, only traces for the second.

Further research in the acid, and alkaline, and salt hemolysis of the blood of the different species was taken up in October, 1914, in the Department of Preventive Medicine at the Harvard Medical School. The results of this work are here presented.

PLAN OF THE WORK

The plan of this research was, first, to determine the percentage of ammonia, sodium hydroxid, hydrochloric acid, and other inorganic and organic compounds, and salts, necessary to cause complete hemolysis of 1% blood-cell suspensions of different species in 15 minutes; second, to determine a chemical index from an arbitrary time index for each of these species; third, to apply these established indices as a means of identifying blood from different species; fourth, to compare the index of the normal with that of the pathologic blood specimen.

The scope of the work as presented in this paper was to establish a chemical standardization for definite hemolytic time indices of dif-

ferent species. This work is to be considered simply preliminary; the chemical indices established by this work being used as a basis, however, it is hoped that by further research our knowledge of the physico-chemical variation in normal and pathologic blood may be extended. Moreover, may not further work along the lines herein reported be applied to a study of living and dead blood, especially as to the ionic content, and the relation of this to the formation of fibrin?

Technic

The blood used in these experiments was obtained by several methods, either by severing the jugular and carotid, or by drawing the blood into a syringe directly from the heart. (The blood should be drawn into a clean container, or, if the syringe be used, this should be rinsed with physiologic salt solution, and the piston be drawn back and driven forcibly in order to expel the last drop of the liquid.) During anesthesia rabbits, guinea-pigs, dogs, monkeys, etc., may be bled from the heart without endangering the life of the animal. Blood from the severed neck vessels of the sheep, ox, chicken, and hog was obtained at the abattoir. In obtaining blood from the frog, rat, and turtle, the animal was first anesthetized; then after exposure of the heart, this was pierced with a pipet, and the blood drawn and finally transferred to a clean container. Blood from man was drawn from the median cephalic vein in the usual manner.

Unless otherwise stated, all blood specimens were defibrinated by gentle whipping with a clean glass rod. The defibrinated blood used in these experiments was in the proportion of 1 c.c. to 99 c.c. of 0.9 % NaCl.

The procedure used in carrying out the various tests was as uniform as possible. The tubes (7 by 80 mm.), which of course were clean, immediately before using were rinsed with 0.9% NaCl solution and drained. They were then placed in the rack, which provides for 12 pairs of tubes. The chemical hemolysin was next measured into the tubes from a double-right-angled pipet. This pipet was standardized to 50 drops of water to the cubic centimeter. It was essential that the drops should be of uniform size, and this uniformity was established by the use of the double-right-angled pipet. To measure accurately 50 drops to the cubic centimeter the dropper arm of the pipet had to be in a perpendicular position; otherwise the size of the drop was increased. Of course any number of drops per cubic centimeter might have been adopted as a standard, but it was essential that these should be uniform and that a standard should be adopted for the entire series of experiments.

Then by adding 1 c.c. of the blood cell suspension to each tube in the series, the immediate mixing of the blood cell suspension with the chemical already in the tube was facilitated somewhat; each pair of tubes was then gently shaken so as to complete the mixing of their contents. Finally the rack was placed in a water-bath at 38 C. (These racks were so constructed that the tubes were immersed to a depth of 2 c.c. with the result that the contents of the tubes were beneath the surface of the water in the bath.) The time of complete hemolysis in each tube in the series was recorded. This series of recorded results represented the time indices, and the equivalent chemical percentages of these indices could then be determined.

The hemolytic time indices of a series of chemical percentages for a given species could be determined only by trial. If, for example, from the standard

pipet from 1 to 12 drops of a definite normality of acid or alkali were placed in a series of 12 tubes and to each of these was added 1 c.c. of a given blood suspension, the time required for complete hemolysis for each percentage of acid or alkali would represent the time index. To illustrate, the hemolysis might be complete in all tubes in a few minutes; or the reaction might be complete in Tube 12 in 5 minutes, and in Tube 1 in 50 minutes. On the other hand, to obtain a given time index, especially a definite time for a certain tube in the series, the experiment became one of considerable difficulty. Moreover, with certain species numerous trials were necessary before the standard time index was determined. This was true particularly with the goat-, dog-, and horse-blood suspensions, each of these requiring many trials.

The series of experiments reported in this paper dealt with the hemolytic effect of the following chemical solutions: ammonia, sodium and potassium hydrate, hydrochloric and sulfuric acid, and acetic acid. These were made up accurately on a normality basis in 0.9% NaCl solution, and, as will be shown later, a sufficient amount of NaCl was added to the standard acid or alkali to render the final solution exactly 0.9% NaCl. To illustrate, 200 c.c. of commercial ammonia were added to 900 c.c. of 0.9% NaCl solution, then 1.8 grams NaCl were added to this solution in order to bring the total NaCl content up to 0.9%. Finally this was titrated, and a sufficient amount of 0.9% NaCl was added to render the ammonia content 3N. NH_3. This salt content, of course, decreased the acid and alkaline content to a lower point than it would have been in a water solution. Nevertheless, this procedure should be taken as a standard method in this work in order to maintain a uniform salt content in all subsequent dilutions with blood cell suspensions. The chemical indices as well as other chemical considerations are given on a percentage basis, and the tables applied for transposing the standard solutions to their equivalent percentages are here presented.

Should the acid or alkaline solution be made up in water, it is obvious that, if unequal quantities were added to uniform quantities of blood cell suspensions in 0.9% NaCl solution, the salt content would vary. For instance, if one drop of an ammonia solution in water be added to 1 c.c. of a blood cell suspension in 0.9% NaCl solution, the final NaCl content, according to the formula $51 : 50 :: 0.9 : X$, would be 0.88% NaCl. If diluted with 12 drops, it would be, $62 : 50 :: 0.9 : X$, or 0.72% NaCl. These variations in the final salt content have a definite effect on the period of hemolysis. If, on the other hand, the acids and alkalies be in 0.9% NaCl solution, the addition of these to the blood cell suspensions will be accompanied by no decrease in the standard salt percentage.

To ascertain the time index of a certain blood cell suspension the procedure in these experiments was as follows: First, in a series of 12 pairs of tubes 1 drop of the chemical hemolysin was placed in one tube of the first pair (its mate serving as a control), 2 drops in the corresponding tube of the second pair, and so on successively, including the tube of the twelfth pair, which received 12 drops. Second, 1 c.c. of the blood cell suspension was then run into each tube in the series. Third, each pair of tubes was removed from the rack, turned at an angle of 45, and shaken. Fourth, the rack was then placed in

TABLE 1

Table Applied in This Work for Transposing Standard Solutions of NH₃ to Their Corresponding Percentages

		3N	2N	N/1	N/2	N/3	N/4	N/5	N/10	N/15	N/60
1	N/51.........	.1	.066	.033	.016	.011	.0083	.0066	.0033	.0022	.0005
2	N/26.........	.196	.13	.0654	.0327	.0218	.0162	.013	.0065	.0043	.001
3	N/17.6.......	.288	.192	.0964	.0432	.0321	.0241	.0193	.0096	.0064	.0016
4	N/13.5.......	.378	.252	.126	.063	.042	.031	.025	.0126	.0084	.0021
5	N/11.........	.463	.308	.154	.077	.051	.038	.031	.0154	.01	.0025
6	N/9.3.........	.548	.364	.182	.091	.061	.054	.036	.018	.012	.003
7	N/8.2.........	.621	.414	.207	.103	.069	.052	.041	.021	.013	.0034
8	N/7.2.........	.708	.472	.236	.118	.079	.059	.047	.023	.015	.0039
9	N/6.5.........	.783	.522	.261	.130	.087	.065	.052	.026	.017	.0043
10	N/6.........	.849	.566	283	.141	.094	.071	.056	.028	.018	.0047
11	N/5.5.........	.927	.618	.308	.154	.103	.077	.062	.031	.02	.0051
12	N/5.16.......	.978	.652	.326	.163	.109	.081	.065	.034	.022	.0054

TABLE 2

Table Applied in This Work for Transposing NaOH Molecular Solutions to Their Corresponding Percentages

		N/1	N/10	N/12	N/15	N/25	N/28	N/30	N/35	N/50	N/90
1	N/51.........	.0784	.0078	.0065	.0052	.0031	.0028	.0026	.0022	.0015	.0009
2	N/26.........	.153	.0153	.012	.01	.0061	.0055	.0051	.0043	.003	.0017
3	N/17.6.......	.227	.0227	.018	.015	.0093	.0081	.0075	.0064	.0043	.0025
4	N/13.5.......	.2962	.0296	.024	.019	.0118	.0106	.0098	.0084	.0058	.0032
5	N/11.........	.3636	.0363	.03	.024	.0145	.013	.012	.0104	.0072	.004
6	N/9.3.........	.43	.042	.035	.028	.0172	.0155	.0143	.0122	.0086	.0048
7	N/8.2.........	.488	0488	.04	.032	.019	.0173	.016	.0139	.0096	.0054
8	N/7.2.........	.555	.0555	.046	.037	.022	.0198	.018	.0157	.011	.0061
9	N/6.5.........	.615	.0615	.051	.041	.024	.0221	.0205	.0175	.0122	.0068
10	N/6.........	.666	.0666	.055	.044	.026	.024	.0222	.019	.0132	.0074
11	N/5.5.........	.727	.0727	.06	.048	.029	.0261	.0242	.0208	.0144	.008
12	N/5.16.......	.775	.0775	.064	.052	.031	.028	.0258	.0221	.0154	.0086

TABLE 3

Table Applied in This Work for Transposing HCl Molecular Solutions to Their Corresponding Percentages

		N/50	N/75	N/80	N/100	N/120	N/150
1	N/51.......................	.0014	.0009	.0008	.0007	.0006	.0004
2	N/26.......................	.0028	.0018	.0017	.0014	.0011	.0009
3	N/17.6.......................	.0041	.0027	.0025	.002	.0017	.0015
4	N/13.5.......................	.0054	.0035	.0033	.0027	.0022	.0017
5	N/11.......................	.0066	.0043	.0041	.0033	.0027	.0021
6	N/9.3.......................	.0079	.0052	.0048	.0039	.0033	.0026
7	N/8.2.......................	.0089	.0058	.0055	.0044	.0037	.0029
8	N/7.2.......................	.01	.0066	.0062	.005	.0042	.0033
9	N/6.5.......................	.0112	.0072	.0068	.0055	.0046	.0036
10	N/6.......................	.012	.0079	.0074	.006	.005	.0039
11	N/5.5.......................	.013	.0085	.0081	.0065	.0054	.0042
12	N/5.16.......................	.014	.0092	.0086	.007	.0058	.0046

Explanation of Tables 1, 2, and 3

The first vertical row represents the serial tubes in the rack, also the number of drops of the standard solution added: 1 drop to the first tube, 2 drops to the second tube, etc., to 12 drops to the twelfth tube. The second row states the normality coinciding with the number of drops added to an N solution. The following successive rows represent the percentages of the chemical corresponding to the normality indicated at the top of each column, and to the number of drops in the series.

the water-bath, and finally a record made of the time of the completion of hemolysis in each tube.

As the rapidity of hemolysis depends on the percentage of the hemolytic agent, it was found that, with the exception of hydrochloric acid, Tube 12, i. e., the one containing the highest percentage, hemolyzed first in the series and Tube 1 last.

If preliminary tests be made on a given species, using as the hemolytic agent percentages of a certain chemical, the time indices for the series of tubes may be comparatively short—1 minute in the twelfth tube, and 10 minutes in the first tube. As has been stated, the first problem was to determine the chemical percentages necessary to complete hemolysis in 15 minutes. Furthermore, it was found, as the work progressed, that the fifteen-minute hemolytic system should occur in the fifth tube in the series. This is advisable in order that the factor of dilution of the blood components—acid, alkaline, and neutral salt ions, serum, and cells—with the hemolytic agent be kept as nearly uniform as possible for the fifteen-minute hemolytic system. If the fifteen-minute hemolytic system be in the fifth tube for one test, and in the twelfth tube for another, the variation in the percentages of blood components would be 0.909% in the fifth tube, and 0.806% in the twelfth tube; this variation in dilution would cause an appreciable lack of uniformity in results.

As in the test described, if all tubes are hemolyzed in 10 minutes, it is obvious that the chemical hemolytic agent should be reduced. Inasmuch as the number of drops remains the same in each set of tubes, it is necessary to reduce the percentage or normality of the standard chemical solution. As no exact rule as yet appears applicable to all species for the reduction of percentage or normality, that normality which produces complete hemolysis in the fifth tube in the series in 15 minutes can be ascertained only by trial. After having found the normality which will give hemolysis in the fifth tube in 15 minutes, the chemical percentages can be determined for each tube in the series, and the recorded results serve as the hemolytic time indices.

It was necessary to find whether or not these percentages would give the same time indices for numerous specimens, each taken from a different animal of the same species. (Unless otherwise stated, the blood cell suspensions were from normal animals.)

That this work at the present stage of its progress is subject to minor errors, must be taken into consideration. These, however, may be eliminated as the technical details are perfected. Small quantities of both chemicals and blood cell suspensions were used, and the cell suspension was only 1%. The difficulty of accurately measuring 1 c.c. from a 10 c.c. pipet accounts, at least to some extent, for the slight variation in the time indices for the same species and the same chemical percentages. This is particularly true of those high chemical dilutions in the first tubes of the series; while it is not of great importance with the lower dilutions. Should the amount of cell suspension be increased to several cubic centimeters with a corresponding increase in chemical, this alone would tend to give more accurate results. Furthermore, should the percentage of cell suspension be increased from 1%—that adopted in this work—to 5 or 10%, it is obvious that with the same time indices, there would be a relatively greater variation in the chemical percentages. Primarily, the object in using a 1% cell suspension was that, if with this low percentage, a difference in species could be shown, then, with the higher percentages, there could be no question concerning this point.

The difference between the chemical percentages for the same time indices of the horse and the mule was not great for the 1% blood cell suspension, but when the suspension was increased to 10%, the difference at once became more evident (Charts VI and VII). Tho this was true for these two animals, it is not necessarily so for other species, for there is no exact proportional relation between the percentage of the blood cells, the chemical percentage, and the time of complete hemolysis. That is to say, 1% defibrinated blood suspensions may with the same chemical index give different time indices, but should both suspensions be increased to 10% both may give the same time index, or the one giving the shorter time index with the 1% may even give the longer index with the 10% blood suspension. In short, the law of definite proportions applies to this work to a limited extent only. It may be stated, also, that this law does not apply to the chemical and blood cell percentages. A 1% cell suspension gives definite time indices for a fixed series of chemical percentages; when the cell suspension is increased to 10% it is found that less than 10 times the chemical percentage is required to give approximately the same time index. The constituents of the blood serum may be responsible for the irregular proportional relationship, and may it not be assumed

TABLE 4

TABLE OF CHEMICAL EQUIVALENTS IN PERCENTAGE OF A 15-MINUTE HEMOLYTIC SYSTEM FOR
1% BLOOD CELL SUSPENSIONS OF DIFFERENT SPECIMENS FROM THE SAME SPECIES

Animal	Drops	NH₃	Percent-age	Drops	NaOH	Percent-age	Drops	HCl	Percent-age
Goat 1.............	6	3N	.548	4	N/10	.0296	7	N/120	.00325
2.............	6	3N	.548	4	N/10	.0296	7	N/120	.00325
3*......... ..	5	3N	.463	4	N/10	.0296	7	N/124	.0036
Cow (Bovine) 1.....	6	2N	364	4	N/12	.024	3	N/80	.0026
2.....	6	2N	.364	4	N/12	.024	3	N/80	.0026
3.....	6	2N	.364	4	N/12	.024	3	N/80	.0026
4.....	6	2N	.364	4	N/12	.024	3	N/80	.0026
5.....	4	3N	.378	4	N/12	.024	3	N/80	.0026
6.....	4	3N	.378	4	N/12	.024	3	N/80	.0026
7.....	4	3N	.378	4	N/12	.024	2	N/80	.0018
8.....	4	3N	.378	4	N/12	.024	2	N/80	.0018
9.....	4	3N	.378	4	N/12	.024	3	N/80	.0026
10.....	4	3N	.378	4	N/12	.024	3	N/80	.0026
11.....	4	3N	378	4	N/12	.024	3	N/80	.0026
Sheep 1.............	7	2N	.414	6	N/15	.028	6	N/90	.0044
2.............	4	3N	.378	5	N/15	.024	8	N/150	.0033
3.............	4	3N	.378	5	N/15	.024	10	N/150	.0039
4.............	4	3N	.378	5	N/15	.024	9	N/150	.0036
5.............	4	3N	.378	5	N/15	.024	9	N/150	.0036
6.............	4	3N	.378	5	N/15	.024	6	N/100	.0039
Deer 1.............	6	2N	.364	6	N/25	.0172	7	N/75	.0058
Negro 1.............	11	N	.308	7	N/25	.019	7	N/100	.0044
2.............	11	N	.308	7	N/25	.019	7	N/100	.0044
3.............	5	2N	.308	6	N/25	.0172	7	N/100	.0044
4.............	5	2N	.308	6	N/25	.0172	7	N/100	.0044
Elephant 1..........	5	2N	.308	7	N/25	.019	2	N/75	.0018
Cat 1...............	3	3N	.288	5	N/15	.024	8	N/100	.005
2...............	3	3N	.288	5	N/15	.024	8	N/100	.005
3...............	3	3N	.288	5	N/15	.024	8	N/100	.005
4...............	3	3N	.288	6	N/15	.028	10	N/100	.006
5...............	3	3N	.288	5	N/15	.024	8	N/100	.005
6...............	3	3N	.288	6	N/15	.028	10	N/100	.006
7...............	3	3N	.288	5	N/15	.024	8	N/100	.005
8...............	3	3N	.288	4	N/15	.019	9	N/100	.0056
9...............	4	3N	.378	5	N/15	.024	4	N/100	.0026
10...............	3	3N	288	4	N/15	.019	5	N/100	.0033
11†...............	3	3N	.288	4	N/15	.019	5	N/150	.0021
12†...............	3	3N	.288	4	N/15	.019	5	N/150	.0021
13†...............	3	3N	.288	4	N/15	.019	5	N/150	.0021
Monkey 1..........	6	N	.182	7	N/35	.0139	6	N/100	.0039
2..........	7	N	.207	7	N/35	.0139	5	N/100	.0033
3..........	7	N	.207	7	N/35	.0139	6	N/100	.0039
4..........	7	N	.207	7	N/35	.0139	6	N/100	.0039
5..........	7	N	.207	7	N/35	.0139	5	N/100	.0033
6‡..........	4	N	.207	4	N/35	.0084	6	N/75	.0052
Caucasian 1........	3	2N	.192	7	N/28	.0155	4	N/50	.0054
2........	6	N	.182	7	N/28	.0155	4	N/50	.0054
3........	6	N	.182	7	N/28	.0155	6	N/75	.0052
4........	...	......	.....	7	N/28	.0155	6	N/75	.0052
5........	5	N	.1546	6	N/25	.0172	6	N/80	.0048
6........	5	N	.1546	6	N/25	.0172	7	N/80	.0055
7........	5	N	.1546	6	N/25	.0172	6	N/80	.0048
8........	6	N	.182	6	N/25	.0172	6	N/75	.0052
9........	5	N	.1546	5	N/25	.0144	6	N/75	.0052
10........	5	N	.1546	6	N/25	.0172	6	N/75	.0052
11........	5	N	.1546	6	N/25	0.172	6	N/75	.0052
12........	5	N	.1546	5	N/25	.0144	6	N/75	.0052
13........	6	N	.182	6	N/25	.0172	9	N/100	.0055
14........	6	N	.182	6	N/25	.0172	9	N/100	.0055

* Sensitized to rabbit red blood cells.
† Two-day-old kittens.
‡ Case of cage paralysis.

TABLE 4—Continued

TABLE OF CHEMICAL EQUIVALENTS IN PERCENTAGE OF A 15-MINUTE HEMOLYTIC SYSTEM FOR 1% BLOOD CELL SUSPENSIONS OF DIFFERENT SPECIMENS FROM THE SAME SPECIES

Animal	Drops	NH₃	Percentage	Drops	NaOH	Percentage	Drops	HCl	Percentage
Swine 1	6	N	.182	6	N/28	.0155	6	N/75	.0052
2	6	N	.182	6	N/28	.0155	6	N/75	.0052
3	6	N	182	6	N/28	.0155	6	N/72	.0052
4	6	N	.182	6	N/30	.0143	6	N/75	.0052
5	5	N	.154	6	N/30	.0143	6	N/75	.0052
6	5	N	.154	6	N/30	.0143	6	N/75	.0052
7	5	N	.154	6	N/30	.0143	6	N/75	.0052
8	5	N	.154	6	N/30	.0143	6	N/75	.0052
9	5	N	.154	6	N/30	.0143	6	N/75	.0052
10	5	N	.154	6	N/30	.0143	6	N/75	.0052
Frog 1	4	N	.126	5	N/25	.0145	4	N/75	.0035
2	4	N	.126	5	N/25	.0145	4	N/75	.0035
3	4	N	.126	5	N/25	.0145	4	N/75	.0035
4	4	N	.126	5	N/25	.0145	4	N/75	.0035
Pigeon 1	4	N	.126	7	N/30	.016			
2	4	N	.126	7	N/30	.016			
Chicken 1	6	N/2	.091	6	N/25	.0172			
2	7	N/2	.103	6	N/25	.0172			
3	5	N/1.75	.088	5	N/25	.0145			
Rabbit 1	6	N/2	.091	6	N/35	.0122	4	N/40	.0067
2	5	N/2	.077	6	N/35	.0122	4	N/40	.0067
3	4	N/2	.063	6	N/35	.0122	5	N/50	.0066
4	7	N/3	.069	6	N/35	.0122	5	N/50	.0066
5	7	N/3	.069	6	N/35	.0122	5	N/50	.0066
6	4	N/1.75	.066	6	N/35	.0122	5	N/50	.0066
7	4	N/2	.063	5	N/35	.01	5	N/50	.0066
8	4	N/2	.063	5	N/35	.01	5	N/50	.0066
9§	2	N/1.75	.037	6	N/35	.0122	4	N/50	.0054
10‖	2	N/2	.0327	4	N/35	.0084	8	N/50	.01
Guinea-pig 1	6	N/3	.061	5	N/30	.012	4	N/75	.0035
2	6	N/3	.061	5	N/30	.012	4	N/75	.0035
3	6	N/3	.061	...	......	.....	4	N/75	.0035
4	6	N/3	.061	...	......	.....	4	N/75	.0035
5	4	N/2	.0629	7	N/50	.0099	5	N/100	.0033
6	4	N/2	.0629	7	N/50	.0099	5	N/100	.0033
7	4	N/2	.0629	8	N/50	.0104	11	N/200	.0033
8	4	N/2	.0629	8	N/50	.0104	11	N/200	.0033
9	4	N/2	.0629	8	N/50	.0104	11	N/200	.0033
10	4	N/2	.0629	8	N/50	.0104	11	N/200	.0033
11	4	N/2	.0629	8	N/50	.0104	5	N/100	.0033
12	4	N/2	.0629	8	N/50	.0104	5	N/100	.0033
Turtle 1	3	N/2	.043	4	N/35	.0084	3	N/75	.0027
2	3	N/2	.043	3	N/35	.0064	4	N/75	.0035
3	3	N/2	.043	4	N/35	.0084	3	N/75	.0027
4	3	N/2	.043	4	N/35	.0084	3	N/75	.0027
5	3	N/2	.043	4	N/35	.0084	...	......	.....
6	3	N/2	.043	3	N/35	.0064	...	......	.....
Brown Rat 1	4	N/3	.042	4	N/25	.0118	4	N/80	.0033
2	4	N/3	.042	4	N/25	.0118	4	N/80	.0033
3	4	N/3	.042	4	N/25	.0118	4	N/80	.0033
4	4	N/3	.042	4	N/25	.0118	4	N/80	.0033
5	4	N/3	.042	4	N/25	.0118	4	N/80	.0033
6	4	N/3	.042	4	N/25	.0118	4	N/80	.0033
7	6	N/4	.045	4	N/35	.0084	5	N/75	.0043
8	6	N/4	.045	4	N/35	.0084	5	N/75	.0043
9	6	N/4	.045	4	N/35	.0084	4	N/75	.0035
10	6	N/4	.045	4	N/35	.0084	4	N/75	.0035
12	6	N/4	.045	4	N/35	.0084	4	N/75	.0035
13	6	N/4	.045	4	N/35	.0084	4	N/75	.0035

§ Severe case of so-called snuffles.
‖ Streptococcus septicemia—see chart.

TABLE 4—*Continued*

Table of Chemical Equivalents in Percentage of a 15-Minute Hemolytic System for
1% Blood Cell Suspensions of Different Specimens from the Same Species

| Animal	Drops	NH$_3$	Percentage	Drops	NaOH	Percentage	Drops	HCl	Percentage
White Rat 1........	5	N/4	.038	5	N/50	.0072	5	N/75	.0043
2........	5	N/4	.038	5	N/50	.0072	5	N/75	.0043
3........	5	N/4	.038	5	N/50	.0072	5	N/75	.0043
4........	5	N/4	.038	5	N/50	.0072	5	N/75	.0043
Mule 1..............	6	N/15	.012	4	N/28	.0106	6	N/100	.0039
2..............	8	N/15	.015	5	N/28	.013	6	N/100	.0039
3..............	6	N/15	.012	5	N/28	.013	6	N/100	.0039
4..............	7	N/15	.013	5	N/28	.013	6	N/100	.0039
Horse 1.............	4	N/15	.0084	5	N/28	.013	5	N/100	.0033
2.............	5	N/15	.0103	5	N/28	.013	5	N/100	.0033
3.............	5	N/15	.0103	5	N/28	.013	5	N/100	.0033
4.............	4	N/15	.0084	5	N/28	.013	5	N/100	.0033
5.............	5	N/15	.0103	5	N/28	.013	5	N/100	.0033
6.............	4	N/15	.0084	5	N/28	.013	5	N/100	.0033
Bear 1..............	5	N/60	.0025	3	N/50	.0045	7	N/100	.0044
Dog 1..............	5	N/60	.0025	3	N/90	.0025	6	N/100	.0039
2..............	5	N/60	.0025	3	N/90	.0025	6	N/100	.0039
3..............	5	N/60	.0025	3	N/90	.0025	6	N/100	.0039
4..............	4	N/60	.0021	5	N/120	.003			
5..............	4	N/60	.0021	5	N/90	.004	5	N/75	.0043
6..............	4	N/60	.0021	5	N/90	.004	5	N/100	.0033
7..............	4	N/60	.0021	5	N/90	.004	5	N/80	.0041
8..............	4	N/60	.0021	5	N/90	.004	4	N/80	.0033
9..............	5	N/60	.0025	3	N/90	.0025	4	N/80	.0033
10..............	5	N/60	.0025	4	N/90	.0032	4	N/80	.0033
11..............	4	N/60	.0021	4	N/90	.0032	4	N/80	.0033
12..............	4	N/60	.0021	3	N/90	.0025	6	N/100	.0039
13..............	4	N/60	.0021	4	N/90	.0032	6	N/100	.0039
14..............	5	N/60	.0025	3	N/90	.0025	6	N/100	.0039
15..............	4	N/60	.0021	3	N/90	.0025	7	N/100	.0044
16..............	3	N/90	.0011	4	N/90	.0032	9	N/120	.0046
17..............	3	N/90	.0011	4	N/90	.0032	9	N/120	.0046
18..............	3	N/90	.0011	4	N/90	.0032	8	N/120	.0042
19..............	3	N/90	.0011	4	N/90	.0032	8	N/120	.0042
20¶..............	4	N	.126	5	N/30	.012	4	N/75	.0035
21¶..............	6	N	.182	4	N/30	.0098	6	N/80	.0048

Operative case—see chart.

that by using a cell suspension free from serum constituents there
would be presented a relation between the percentage of cell suspen-
sion, chemical percentage, and time index which agrees, at least more
nearly, with the law of multiple proportions?

Table 4 gives the chemical equivalents in percentage of a fifteen-
minute hemolytic system for a 1% blood cell suspension for different
specimens of the same species as designated.

Table 5 gives the equivalent chemical percentages for the fifteen-
minute hemolytic systems in the species tested.

The fifteen-minute hemolytic system and its chemical equivalents
for the various species having been considered, the next point for dis-

CHART I

CHART SHOWING THE CHEMICAL (NH_3——— $NAOH$—— HCL—·—)
EQUIVALENTS FOR THE FIFTEEN MINUTE HEMOLYTIC
SYSTEM OF ONE PER CENT BLOOD SUSPENSIONS
FOR THE SPECIES INDICATED
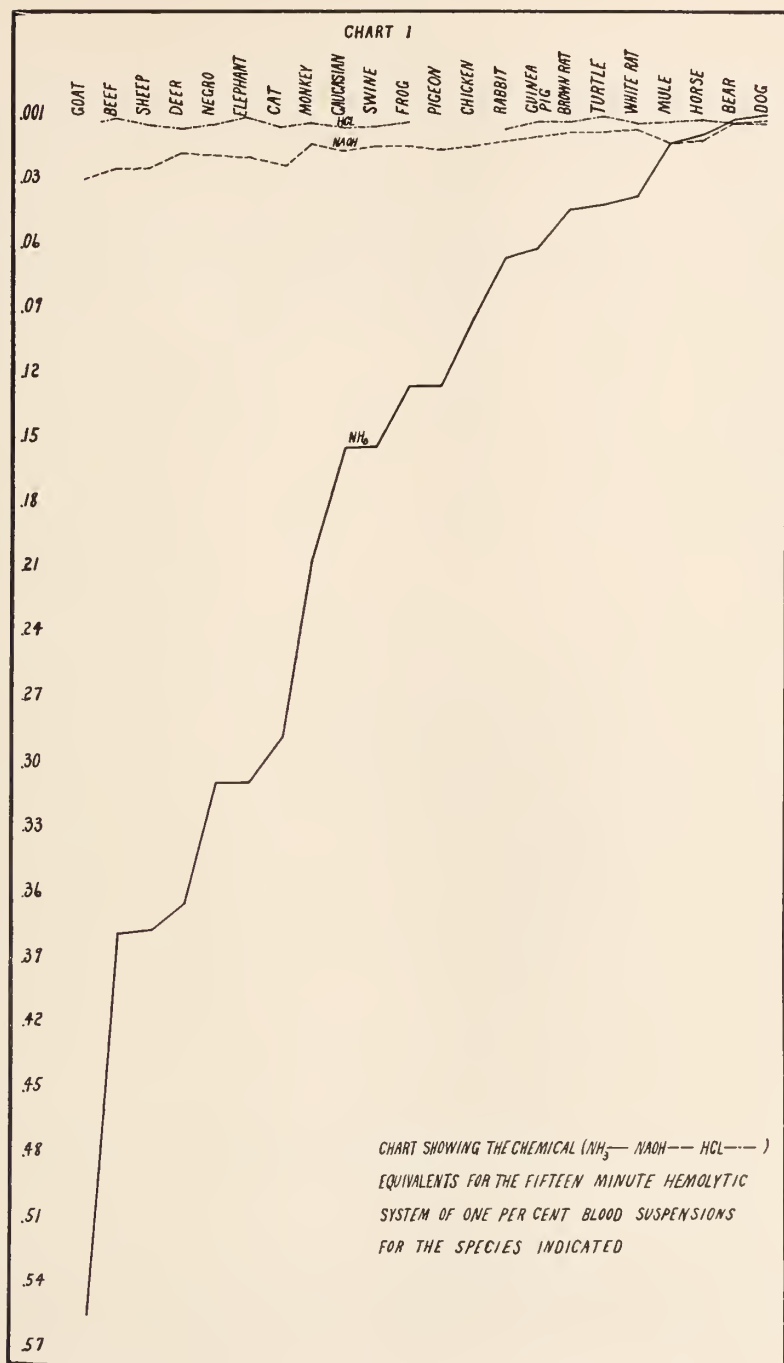
cussion would seem to be the time indices—whatever they chance to be from 3 to 140 minutes—of the chemical hemolysin for the various species. The tabulations of the time indices of the blood of the horse

CHART II

THE NAOH —— AND HCL ––– FIFTEEN MINUTE
HEMOLYTIC INDICES FOR SPECIES INDICATED
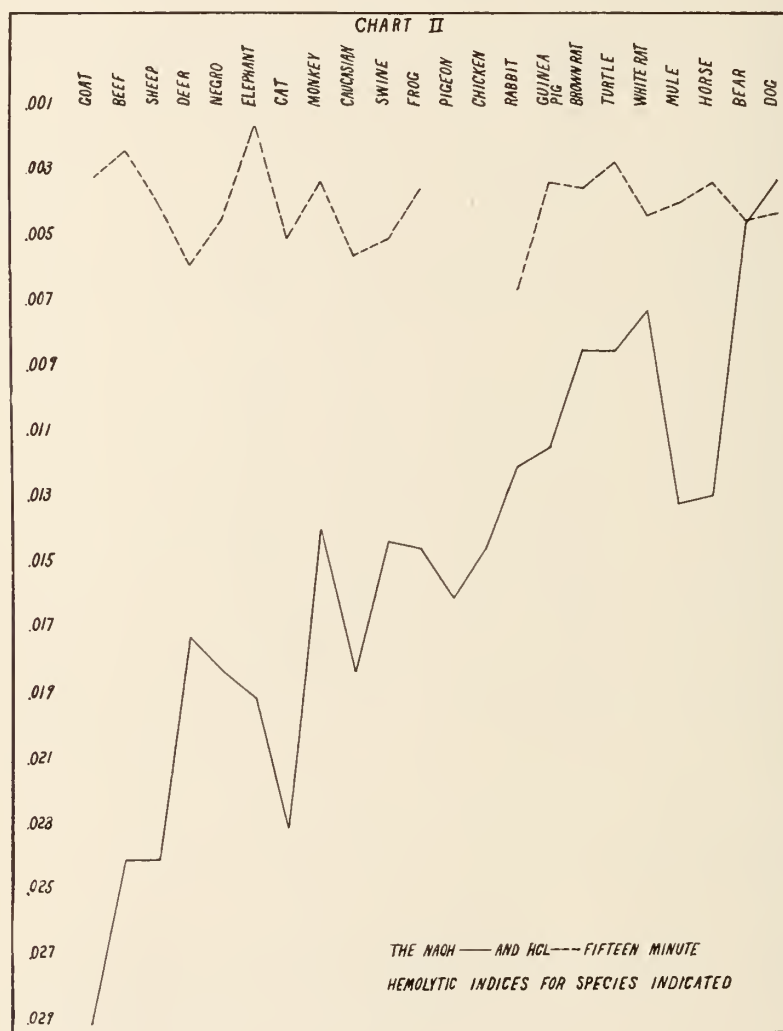
(6 specimens, Tables 6 and 7) and of the monkey (3 specimens, Tables 8, 9, and 10) are here presented in order to illustrate the degree of variation between different specimens of the same species.

TABLE 5

SUMMARY OF THE TABULATIONS IN TABLE 4

Species	NH₃ %	NaOH %	HCl %
Goat	.548	.029	.0032
Beef	.378	.024	.0023
Sheep	.377	.024	.0039
Deer (Virginia White Tail)	.364	.0172	.0058
Negro	.308	.0182	.0044
Elephant (Indian)	.308	.019	.0015
Cat	.288	.023	.005
Monkey (Rhesus)	.207	.0139	.0033
Caucasian	.154	.0162	.0055
Swine	.154	.0143	.005
Frog	.126	.0145	.0035
Pigeon	.126	.016	.....
Chicken	.094	.0145	.....
Rabbit	.066	.012	.0066
Guinea-pig	.0629	.0104	.0033
Brown rat	.045	.0084	.0035
Turtle	.043	.0084	.0027 Incomplete
White rat	.038	.0072	.0043
Mule	.013	.0131	.0039
Horse	.009	.0129	.0033
Bear (American Brown)	.0025	.0045	.0044
Dog	.0011	.0032	.0042

TABLE 6

THE TIME INDICES AND THEIR CORRESPONDING NH₃ PERCENTAGES FOR 6 SPECIMENS OF 1% DEFIBRINATED HORSE BLOOD IN 0.9% NaCl SOLUTION

NH₃ %	Specimen A Time in Minutes	NH₃ %	Specimen B Time in Minutes	Specimen C Time in Minutes	Specimen D Time in Minutes	Specimen E Time in Minutes	Specimen F Time in Minutes
.0033	55	.0022	..	82	85	80	..
.00654	25	.0043	45	38	32	40	35
.00964	15	.0064	30	27	22	28	17
.0126	12	.0084	20	18	15	17	14
.0154	10	.0106	15	15	10	15	12
.0182	8	.012	14	13	8	11	10
.0207	7	.013	12	11	7	9	8
.0236	6	.015	12	10	..	8	7
.0268	5	.017	11	9	6	..	..
.0285	4	.018	9	8	..	..	6
.0308	4	.02	8	8	..	..	..
.0326	4	.022	7	7	5	6	5

In consideration of the high dilution of the chemical hemolysin it is to be noted that there is but slight variation in the time indices for both the lowest and the highest percentages. Attention is called to the fact that the fifteen-minute hemolytic system occurs in the third pair with specimen A, and in the fourth and fifth in the remaining specimens. In considering the fifteen-minute system of C and D it should be borne in mind that there is only 0.0022% difference in the chemical hemolysin contained in each system. Yet, even in these small percentages, there is but slight variation in the time indices for the

six specimens of horse blood; the conditions being similar for dog's blood—the requirement of small quantities of the hemolysin—it is found that this uniformity also holds true. It requires close observation, however, to determine the end point of hemolysis with NH_3, as it is not so well defined as in the case of NaOH.

TABLE 7

The NaOH Percentages and Their Time Indices for 1% Horse Blood

NaOH %	Specimen A Time in Minutes	Specimen B Time in Minutes	Specimen C Time in Minutes	Specimen D Time in Minutes	Specimen E Time in Minutes	Specimen F Time in Minutes
.0028	..	..	..	..	..	..
.0055	..	..	80	85	..	..
.0081	40	38	39	40	38	30
.0106	19	20	20	20	21	17
.0131	14	15	14	15	16	13
.0155	12	11	11	11	11	10
.0173	10	9	..	10	..	..
.0198	8	8	9	..	..	..
.0221	7	7	6	6	..	..
.024	6	6	..	..	..	..
.0261	6	..	..	..	..	..
.028	5	..	6	5	6	5

The slight variation in the time indices in Table 7 may be explained, for the most part, by errors in measurement. The end-point of hemolysis with NaOH is well defined with all species, and the similarity of the time indices with this chemical is a notable feature.

TABLE 8

The HCl Percentages and the Time Indices for 1% Horse Blood

HCl %	Specimen A Time in Minutes	Specimen B Time in Minutes	Specimen C Time in Minutes	Specimen D Time in Minutes	Specimen E Time in Minutes	Specimen F Time in Minutes
.0027	..	..	..	..	..	..
.0033	17	15	17	19	17	12
.0039	8	8	8	8	6	5
.0044	7	7	6	6	4	4
.005	5	5	4	4	..	..
.0055	4	4	..	..	..	..
.006	4	4	4	4	3	3
.0065	..	..	..	..	..	..
.007	..	..	..	..	..	..

The hemolysis of horse blood with HCl (Table 8) breaks off abruptly at 0.0033%, and it is found that even with an exposure of several hours no hemolysis appears in the 0.0027% specimens. Reference to the HCl hemolytic diagram shows that the indices do not extend over a period of more than 15 to 30 minutes. This short index

is probably due to the acid neutralization of the alkalinity of the blood serum, with the formation of neutral salts; the latter prevents hemolysis in some instances much longer than does 0.9% NaCl solution.

TABLE 9

The Time Indices and Their Chemical Equivalents for 1% Monkey Blood in 0.9% NaCl. The Similarity in Time Indices for Different Specimens is Important

NH₃ %	Specimen A Time in Minutes	Specimen B Time in Minutes	Specimen C Time in Minutes
.033	..	54	52
.0654	40	49	38
.0964	30	30	30
.126	27	26	26
.154	22	20	21
.182	18	17	18
.207	15	15	16
.236	13	13	14
.261	12	12	12
.283	10	11	10
.208	9	10	9
.326	8	9	9

TABLE 10

The Time Indices and Their Chemical Equivalents for 1% Monkey Blood in 0.9% NaOH

NaOH %	Specimen A Time in Minutes	Specimen B Time in Minutes	Specimen C Time in Minutes
.0022	..	..	..
.0043	..	..	..
.0064	56	60	60
.0084	35	35	36
.0104	26	26	26
.0122	20	20	20
.0137	15	15	15
.0157	13	13	14
.0175	12	11	12
.019	10	10	11
.0208	9	9	10
.0221	8	8	9

TABLE 11

The Time Indices and Their Chemical Equivalents for 1% Monkey Blood in 0.9% HCl

HCl %	Specimen A Time in Minutes	Specimen B Time in Minutes	Specimen C Time in Minutes
.002	50	..	..
.0027	28	30	27
.0033	14	19	15
.0039	10	14	10
.0044	7	10	..
.005	6	..	4
.0055	5	5	..
.006	5	..	..
.0065	4	..	..
.007	4	4	3

CONSIDERATION OF CHART III

In Chart III the species tabulated, according to their hemolytic time indices would seem to fall into 4 groupings: Group 1, dog, bear, and horse, reacting with the smallest percentage of NH_3; Group 2, the rodents, and in addition the mule and the turtle, requiring higher percentages of NH_3; Group 3, pigeon, frog, Caucasian, swine, and monkey, next in order as to NH_3 requirement; Group 4, chiefly of herbivora— elephant, cow, deer, sheep, and goat—and with these the negro, chicken, and cat, requiring the highest percentages of NH_3.

It may be interesting to note that there appears to be an appreciable difference between the time index of the horse and that of the mule as determined by the NH_3 hemolysis. Altho these two species have not as yet been differentiated by the biologic tests (precipitin, complement fixation, and specific hemolysins), yet there is for the same time-exposure a definite chemical index for the eight horses as differentiated from that of the four mules presented in this work. The percentage of NH_3, corresponding to the fifteen-minute hemolytic system, for the mule (0.033%) is 3 times greater than that for the horse (0.01%).

There is also a difference between the Caucasian and the negro time indices, as appears in this work with 10 Caucasian specimens and 4 negro specimens. Of the four negroes tested, 2 were full-blood as nearly as could be determined; while the other two presented color which would indicate about one-fourth negro. Unless exhaustive work may demonstrate whether or not the time indices will show proportionally the varying dilutions of admixture of Caucasian and negro blood it would be unwise more than to notice the distinct variation found between the blood of the eight Caucasians and the four negroes here reported.

The NH_3 percentage requirements of the fifteen-minute hemolytic system for the two extreme indices (dog and goat) represent a considerable difference—the percentage for the goat being 504.5 times greater than that for the dog. The possibility is apparent of differentiating by the NH_3 index between species in the different groups. The work, however, has not been sufficiently developed to state positively that animals in the same group, giving different indices, can be identified by these indices alone. It is to be noted that the frog and the pigeon, the Caucasian and swine, and the negro and the elephant give the same NH_3 percentages for the fifteen-minute hemolytic system, but

CHART III

HEMOLYTIC TIME INDICES
AND
THEIR EQUIVALENT NH₃ PERCENTAGES
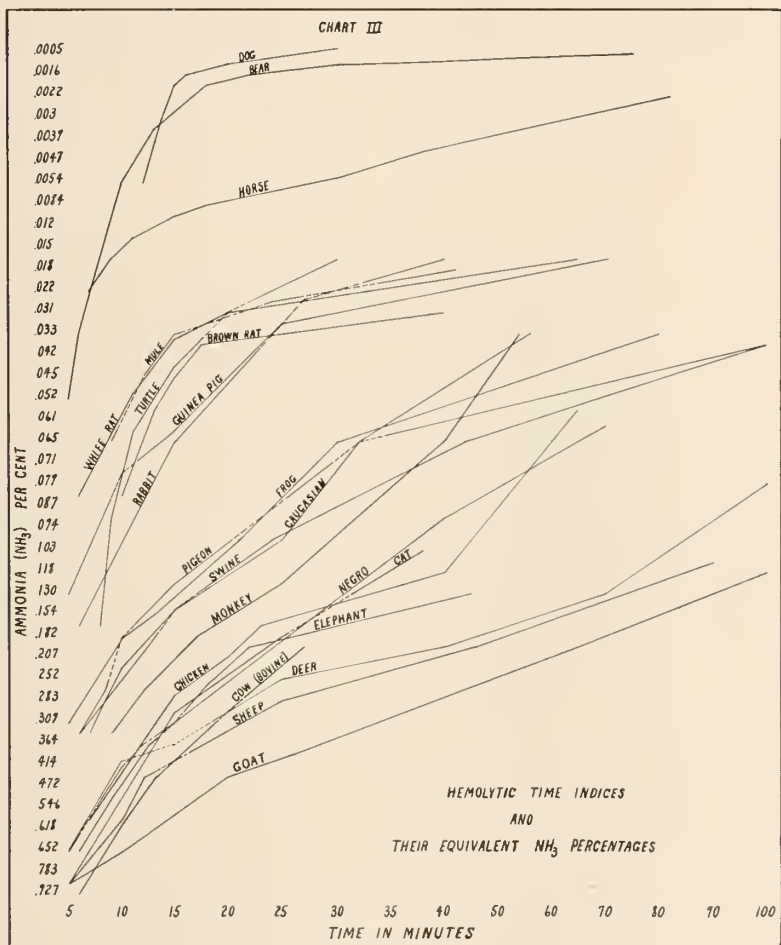
no two of these require the same percentages of NaOH or HCl for the fifteen-minute system. So, for purposes of identification the time indices of all three chemicals should be employed.

In the NaOH chart the separate-group character of the NH_3 chart is practically absent. In both charts, however, the species occupy
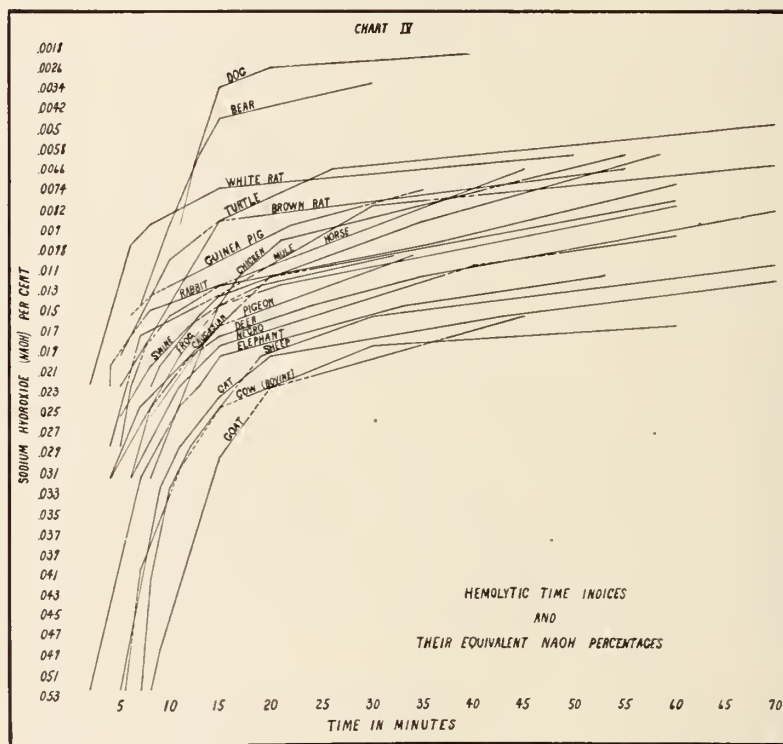
about the same relative positions as to the chemical percentage requirement. In the NaOH chart, as in the NH_3 chart, the dog and the bear react with only traces of the chemical, while the herbivora require the highest percentages.

Attention has been called to the corresponding positions of the NH_3 and NaOH hemolytic time indices in relation to the chemical indices; there would seem to be, however, no special arrangement of

the HCl hemolytic time indices in relation to the alkaline time indices (see Chart V).

A 1% blood suspension from the goat was used for comparing hemolysis by hydrochloric acid with that by sulfuric acid, and hemolysis by sodium, with that by potassium, hydrate. It was found that the time indices were the same for like normalities of the two acids, and also of the two alkalies. For the fifteen-minute system $N/1119.6$ HCl and H_2SO_4 were required. This normality gives 0.00325% for the HCl and 0.00437% for the H_2SO_4. As the same normalities cause hemolysis in the same time limit, it is probable that the reaction is due to the H ion. Like normalities give the same hemolytic indices; consequently, the percentages of HCl and H_2SO_4 must be to each other as their molecular weights:

$$\frac{3.645}{\frac{.00325}{1119.6}} = \frac{4.9}{\frac{.00437}{1119.6}}$$

For the fifteen-minute hemolytic system $N/135$ NaOH and KOH were required. This normality gives 0.029% for the NaOH and 0.041% for the KOH. The hemolysis must depend on the hydroxyl (OH) ion, as the acids depend on the H ion. Here again the percentages of NaOH and KOH must be to each other as their molecular weights:

$$\frac{5.6}{\frac{.041}{135}} = \frac{4.0}{\frac{.029}{135}}$$

CHART V

HEMOLYTIC TIME INDICES AND EQUIVALENT HCL PERCENTAGES

HYDROCHLORIC ACID (HCl) PER CENT

TIME IN MINUTES
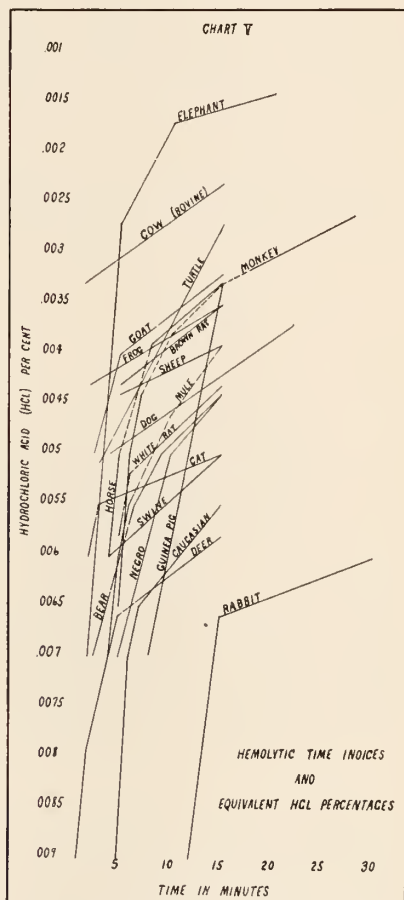
For the fifteen-minute hemolytic system $N/3.66$ NH_3 is required, which gives 0.464%. The normality of NH_3 varies considerably from that of the alkali hydroxids, but this variation probably can be explained by the different constitution of the NH_3 molecule when combined with water.

To determine the effect of the blood serum components—protein, acid, alkaline, and neutral salt ions—on the hemolytic index, it is necessary to find the indices for both the unwashed and the washed blood cell suspensions. To show this influence the following experiments with rabbit and mule blood are given:

The unwashed cell suspension was made up in the usual manner (1 c.c. of defibrinated blood to 99 c.c. of 0.9% NaCl), while the washed cell suspension was prepared by washing by centrifugation 1 c.c. of the defibrinated blood in 75 c.c. of 0.9% NaCl solution 3 successive times. The cells were finally taken up in 99 c.c. of 0.9% NaCl solution.

These suspensions were tested with the results shown in Table 12.

TABLE   12

EFFECT OF BLOOD SERUM COMPONENTS ON THE HEMOLYTIC INDEX

Experiment 1			Experiment 2			Experiment 3		
NH_3 %	Unwashed Suspension	Washed Suspension	NaOH %	Unwashed Suspension	Washed Suspension	HCl %	Unwashed Suspension	Washed Suspension
.016	..	20	.0022	..	..	.002	..	..
.0327	24	15	.0043	..	..	.004	..	13
.0432	21	12	.0064	..	25	.0058	17	9
.063	19	9	.0084	30	14	.0077	15	7
.077	17	7	.0104	19	11	.0098	12	6
.091	15	6	.0122	15	6	.0113	9	5
.103	13	5	.0139	13	5	.0127	7	5
.118	12	4	.0157	9	5	.0143	7	4
.130	11	4	.0175	6	4	.016	6	4
.141	10	3	.019	5	3	.0172	6	4
.154	9	2	.0208	..	..	.0185	5	3
.163	4	2	.0221	..	..	.02	5	3

It will be observed in Experiment 1 that for the fifteen-minute hemolytic system the $NH_3\%$ requirement is for the unwashed suspension almost 3 times greater (0.091%) than for the washed suspension (0.0327%), the washed cells being hemolyzed more readily, and the serum constituents having an inhibiting power of about 66% of the NH_3. The causes of this inhibition will be noted later in this paper.

In Experiment 2 the difference in the NaOH time indices for the unwashed and the washed cell suspensions is not so great as is the

difference in the corresponding NH_3 time indices. There is for the unwashed suspension a requirement of 0.0122% NaOH for the fifteen-minute system; while 0.0083% is required for the washed suspension. These percentages represent a difference of 0.0038% between the two suspensions with NaOH; whereas the difference is 0.0573% between these two suspensions with NH_3.

Another method of reaching the same conclusion in considering the combining power of the serum, or the inhibiting power of its acid or salt ionic content, is to increase the NaOH% in the unwashed series until it is equal to the difference in percentage for the fifteen-minute system in the two suspensions.

Experiment 4 shows the time indices of the unwashed and the washed blood cell suspensions when treated with the two chemical indices (that for the unwashed increased 0.0043%).

TABLE 13
EXPERIMENT 4

NaOH %	Unwashed Suspension	NaOH %	Washed Suspension
.0065	50	.0022	..
.0086	23	.0043	40
.0107	17	.0064	22
.0127	12	.0084	14
.0147	9	.0104	10
.0165	8	.0122	8
.0182	7	.0139	7
.0257	7	.0157	6
.0218	6	.0175	5
.0233	5	.019	4

By increasing the NaOH 0.0043% in the series of unwashed suspensions the time indices of the two suspensions tend to duplicate each other. This result verifies the conclusion in the consideration of Experiment 3; namely, that the combining power of the serum constituents is about 0.0043% NaOH.

Furthermore, if the standard chemical index—from 0.0022% to 0.0221% NaOH—be added to the two suspensions and 0.0043% HCl be added to the washed suspension series, the similarity in the time indices of the two suspensions will again show that the inhibiting power of the serum is equal to about 0.0043% NaOH.

In Experiment 3 it is shown that for a fifteen-minute system 0.0077% HCl is required for the unwashed suspension; while 0.004% is required for the washed suspension. There is then a difference of 0.0037% HCl for the washed and the unwashed suspensions. This is

verified in Experiment 5, in which the acid was increased 0.0042% in the washed series with the result that the time indices were similar for the two suspensions.

This inhibition of hemolysis by serum for the fifteen-minute system to the extent of 0.0042% HCl may be verified a third time by adding its equivalent NaOH% to the washed cell series with the result that the time indices are similar.

It may be stated that the difference in the time indices with the three chemicals varies for different species, and may we not find a sufficient variation for different species to justify the application of this method in a study of the physical chemistry of the blood of different species? The work is not yet sufficiently advanced to state whether or not the inhibiting action of the serum components is due to the power of the protein to combine with the alkali, or to the pro-

TABLE 14

EXPERIMENT 5

HCl %	Unwashed Suspension	HCl %	Washed Suspension
.0062.........................	..	.002.........................	..
.0082.........................	15	.004.........................	13
.01.........................	13	.0058.........................	9
.0119.........................	9	.0077.........................	7
.014.........................	7	.0098.........................	6
.0155.........................	8	.0113.........................	5
.0169.........................	7	.0127.........................	5
.0185.........................	7	.0143.........................	4
.0202.........................	6	.016.........................	4
.0214.........................	5	.0172.........................	3
.0227.........................	5	.0185.........................	3
.0272.........................	4	.02.........................	3

tective film surrounding the cell, or to the action of neutral salt or acid ions. Acid hemolysis may be influenced by any of these factors, together with that of the alkalinity of the serum.

In pursuing this phase of the work it is hoped that by the aid of the red blood cells as an indicator, means may be developed by which the percentage of the serum components can be accurately determined. The work already accomplished in this direction will be reported in a later paper.

The variation in the time indices caused by increasing the NaCl is shown in Experiments 6, 7, and 8 with rabbit blood suspensions.

In Experiment 6 the blood cell suspension in Series A was 1 c.c. defibrinated blood to 99 c.c. 0.9% NaCl solution; whereas, in Series

B, 1 c.c. of the defibrinated blood was made up in 99 c.c. 2.5% NaCl solution. The influence of increasing the NaCl in Series B is noted by comparing the time indices of this series with those of Series A. In Series B the presence of the NaCl accelerated the NH_3 hemolysis in the first two specimens; while the time for the NH_3 hemolysis of the

TABLE 15
Experiment 6

NH₃ %	Series A 0.9 % NaCl	NH₃ %	Series B 2.5 % NaCl
.033...........................	65	.033...........................	25
.0654..........................	20	.0654..........................	19
.0964..........................	15	.0964..........................	20
.126...........................	12	.126...........................	18
.154...........................	11	.154...........................	14
.182...........................	10	.182...........................	11
.207...........................	9	.207...........................	9
.236...........................	8	.236...........................	8
.261...........................	7	.261...........................	7
.283...........................	6	.283...........................	6

corresponding specimens in the physiologic solution was the usual requirement. It should be noted further that there was a retardation of hemolysis in Specimens 3, 4, 5, and 6 of Series B in comparison with the hemolysis in the corresponding specimens in the physiologic salt solution. There is no difference in the indices of the two series in

TABLE 16
Experiment 7

NaOH %	Series A 0.9 % NaCl	NaOH %	Series B 2.5 % NaCl
.0031..........................	..	.0031..........................	..
.0061..........................	38	.0061..........................	35
.0093..........................	18	.0093..........................	19
.0118..........................	12	.0118..........................	13
.0145..........................	10	.0145..........................	11
.0172..........................	9	.0172..........................	10
.019...........................	8	.019...........................	9
.022...........................	7	.022...........................	7
.024...........................	6	.024...........................	6
.026...........................	6	.026...........................	6

the remaining specimens. It is important to note that the hyperiso-tonic salt solution accelerates the hemolysis of certain NH_3 percentages; while with the other percentages of NH_3 there is a retardation.

The increase of the NaCl content influences the NaOH time indices as shown in Experiment 7 (Table 16).

There is but slight variation in the NaOH time indices for the isotonic and the hyperisotonic salt solutions. The acceleration and retardation in hyperisotonic Series B correspond to acceleration and retardation in the NH_3 Series B in Experiment 6.

The effect of hyperisotonicity on the HCl hemolytic time index is shown in Experiment 8 (Table 17).

TABLE 17
Experiment 8

HCl %	Series A 9.9 % NaCl	HCl %	Series B 2.5 % NaCl
.0014	..	.0014	..
.0028	..	.0028	..
.0041	35	.0041	..
.0054	22	.0054	..
.0066	15	.0066	..
.0079	12	.0079	..
.0089	8	.0089	..
.01	7	.01	..
.0112	6	.0112	..
.012	5	.012	..

In Experiment 8 with HCl hemolysis the hyperisotonic (2.5%) condition of the salt solution entirely prevents the diffusion of the hemoglobin. In the last six specimens of Series B, however, there is a change in the appearance of the suspensions. These acquire a yellowish tinge, and the cells have a tendency toward flocculation.

TABLE 18
Experiment 9

NH_3 %	0.9% NaCl	1.3% NaCl	1.5% NaCl	2.0% NaCl
.0022	..	..	..	..
.0043	40	..	..	60
.0064	26	36	42	50
.0084	19	25	28	35
.01	14	23	24	26
.012	12	21	21	20
.013	11	16	19	24
.015	..	14	18	23
.017	9	..	..	22
.018	..	13	16	..
.02	9	..	..	..
.022	8	12	14	16

The varying degrees of inhibition of NH_3 hemolysis of mule-blood suspension obtained by successively increasing the percentages of NaCl are shown in Experiment 9 (Table 18).

It may be noted in this experiment that with the gradual increase in NaCl percentage there is a relative increase in the time indices.

The inhibition of NaOH hemolysis of mule blood by increasing the NaCl solution to 2% is shown in Experiment 10 (Table 19). For the same hyperisotonic salt solution the retardation of NaOH hemolysis is not so great as of NH_3 hemolysis.

TABLE 19
Experiment 10

NaOH %	Series A 0.9% NaCl	Series B 2.0% NaCl
.0028	..	..
.0055	..	..
.0081	32	42
.0106	18	20
.0131	15	16
.0155	12	13
.0173	11	11
.0198	..	..
.0221	7	8
.024	7	7

The influence on HCl hemolysis of increasing the NaCl content of the suspension to 1.26% is shown in Experiment 11. The inhibition of this hemolysis by the increased NaCl is not marked; whereas with rabbit blood, as shown in Series B of Experiment 8, the inhibition is complete.

Attention has already been called to the possibility of increasing the difference between the time indices for certain species by increasing the percentages of cell suspensions tested. Reference to the fifteen-minute hemolytic indices of the 1% blood suspension will show

TABLE 20
Experiment 11

HCl %	Series A 0.9% NaCl	Series B 2.5% NaCl
.0033	..	..
.0039	14	30
.0044	6	6
.005	5	7
.0055	4	7
.006	4	6
.0065	5	5
.007	6	4

that there was a difference of only 0.004% NH_3 between the horse and the mule. Whereas, for the fifteen-minute system, Chart VI shows for the 10% blood suspension of these two species a difference of 0.023% NH_3. For the 1% blood suspension the NaOH fifteen-minute system shows a difference of 0.001%; while for the 10% blood sus-

pension Chart VII shows for the same time system a difference of 0.004% NaOH. In these two species the NH_3 requirement for the 10% blood suspension was 5.6 times greater than that for the 1% blood suspension; and the NaOH, 4 times greater for the 10% than for the 1% suspension.

CHART VI

HEMOLYTIC TIME INDICES
FOR
TEN PER CENT BLOOD SUSPENSIONS
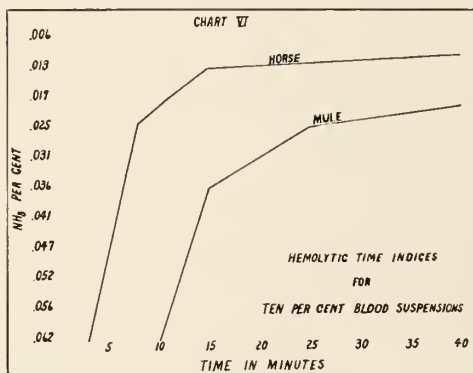
TIME IN MINUTES

A considerable variation will be noted between the time indices for the normal and for the sick monkey. The greatest variation is in the chemical indices for the eight-minute hemolytic system,—a difference in NH_3 of 0.151%; while for the fifteen-minute system this difference is 0.081%, and as the time increases the indices tend to converge,—at the end of 35 minutes there being a difference of only 0.01% (see Chart VIII).

From the experimental viewpoint this decrease in the time index for the sick monkey as compared with that of the normal would seem to be due to a hypo-isotonic condition of the blood. Chart VIII may be compared with Chart X to illustrate the effect of different degrees of iso-tonicity.

In Chart IX there is a shorter time index for the two sick rabbits than for the normal rabbit. There is a slight, but well-defined, variation between A and B; while C shows a consider-able difference from A.

CHART VII

HEMOLYTIC TIME INDICES
FOR
TEN PER CENT BLOOD SUSPENSIONS

TIME IN MINUTES

This difference may possibly be due to an increase in the alkalinity of the blood, or to a decrease in the neutral salt content or acid ions, or to the combining power of the protein.

In Chart X it is shown that for the fifteen-minute hemolytic system 60 times more NH_3 is required for B (one of the infected dogs) than
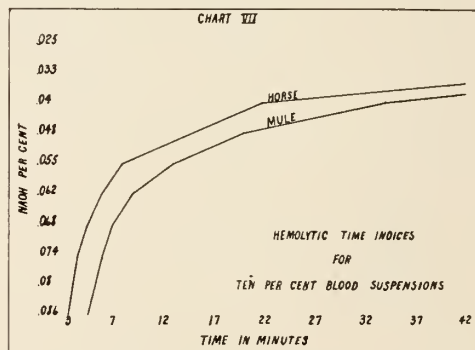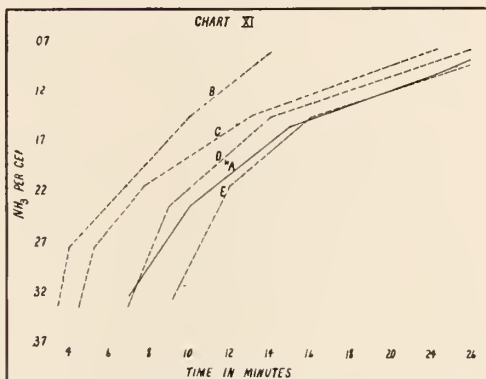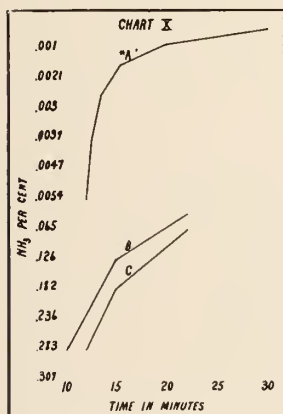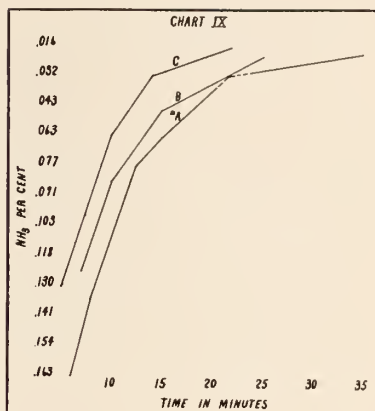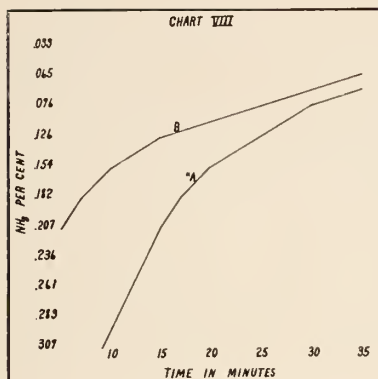
Chart VIII. A = the hemolytic time index with NH_3 for a normal monkey. B = the hemolytic time index with NH_3 for a monkey which had cage paralysis.

Chart IX. A = the hemolytic time index with NH_3 for the normal rabbit. B = the hemolytic time index with NH_3 for a rabbit which was strongly reactive in precipitating beef serum. C = the hemolytic time index with NH_3 for a rabbit infected with the streptococcus of chronic arthritis.

Chart X. A = the hemolytic time index with NH_3 for the normal dog. B and C = the hemolytic time indices with NH_3 for 2 operative dogs in which there was infection following the removal of a section of the intestine.

Chart XI. A = the hemolytic time index and its equivalent NH_3 percentage for normal human blood. B, C, D, and E = the hemolytic time indices and their equivalent NH_3 percentages for 4 cases of scarlet fever. Specimens B and D were obtained from patients during the height of the febrile state; while C and E were taken from patients in a later stage of the disease.

for A (normal dog) ; while for C (the other infected dog) 86.6 more is required than for A.

As shown in Experiments 6 and 9, an increase in the salt content of 1 of 2 specimens, otherwise identical, retards hemolysis, and, to produce the same time indices for both specimens, it would be necessary to increase the percentage of chemical hemolysin in the salted suspension. It may also be stated that by increasing the acidity, the same result is obtained.

Attention is called to the fact that, the chemical index being the same, the time index for the infected dog would be much longer than for the normal dog. In all other pathologic specimens reported in this paper the reverse of this is true.

SUMMARY AND CONCLUSIONS

For the fifteen-minute hemolytic system there is a marked difference in the chemical requirements (NH_3, NaOH, and HCl) for some species, while the difference is not so marked for others. For this system one of the three chemical hemolysins may be of the same percentage for two species; invariably there appears however, a difference in the percentage requirement for either one or both of the other two chemical hemolysins.

The NH_3 hemolytic time indices divide the animals tested into 4 fairly distinct groupings.

The position of the NaOH hemolytic time indices of the different species corresponds closely to that of the NH_3 indices.

There is no special arrangement of the HCl time indices for the different species with relation to the alkaline indices.

By the use of the chemical and hemolytic time indices blood cell suspensions of different species can be identified with a considerable degree of accuracy.

By increasing the percentage of blood from 1% to 10% a greater variation in the time indices of different species may be found.

Alkaline hemolysis may be considered due to the hydroxyl group, and acid hemolysis due to the H ion.

The hemolysis of the red blood cell may be used as an indicator to determine the degree of acidity or alkalinity of certain solutions.

As an indicator the cells are affected by the isotonicity of the blood suspension.

Hemolysis may be an aid in determining molecular weights.

Alkaline hemolysis can be influenced by acids, and acid hemolysis by alkalies. Both acid and alkaline hemolysis can be influenced by the neutral salt content of the suspension.

In most of the specimens tested there was shown a distinct variation between the normal and the pathologic blood of the same species. The time indices in the majority of the latter specimens were increased or decreased in comparison with the normal.

It is possible that the variation between the normal and the pathologic specimens may be accounted for by increased alkalinity, or decreased acidity, or by the variation in the neutral salt content.

THE PHYSICAL CHEMISTRY OF DISINFECTION, I *

J o h n   F.   N o r t o n   a n d   P a u l   H.   H s u

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Various attempts have been made to apply the principles of physical chemistry in the standardization of disinfectants. These, however, have not been wholly successful, altho their entire impracticability has not been demonstrated.

One of the simplest of physico-chemical measurements which can be used to show changes in ion concentrations in solutions, is that of electrical conductivity. Protein degradation may be followed by such a method — the conductivity increasing as the chemical change progresses. With the aid of this principle Schryver and Lessing[1] devised a method for comparing the relative antiseptic values of disinfectants. The rate of chemical change produced in a substrate by bacterial infection was taken as a measure of the vigor of the growth, and the effect on this rate of the presence of any given disinfectant was compared to the effect produced by carbolic acid. The method was designed to measure inhibition rather than sterilization.

It has been shown by Madsen and Nyman[2] and Chick[3] that the killing of bacteria by disinfectants sometimes simulates a monomolecular reaction, in which the bacteria take the place of one of the reacting substances. The other substance, the disinfectant, is present in such excess that its concentration is not materially changed during the reaction. The process may be compared to the inversion of sugar. For example, if the temperature is constant, the reaction may be represented by the equation,

$$- \frac{db}{dt} = kb \tag{1}$$

where "b" represents the number of bacteria present in unit volume after any time, "t," has elapsed and "k" is the velocity constant. After integration the equation has the form,

$$\log \frac{B}{b} = kt \tag{2}$$

in which "B" is the number of bacteria initially present.

Chick,[3] in her work on the laws of disinfection, studied the various phases involved in this reaction, such as the reaction velocity of disinfection, the effect

[1] Jour. Soc. Chem. Ind., 1909, 28, p. 60.

[2] Centralbl. f. Bakteriol., R., 1907, 40, p. 113. Ztschr. f. Hyg. u. Infektionskrankh., 1908, 57, p. 388.

[3] Jour. Hyg., 1908, 8, p. 92.

[4] Jour. Infect. Dis., 1911, 8, p. 27.

on its germicidal action of varying the concentration of a disinfectant, and the influence of temperature upon the velocity of disinfection. Using these principles of physical chemistry, Phelps[4] made the following suggestions to be applied in the standardization of disinfectants. Taking into account the effect of varying the concentration of a disinfectant, we have

$$-\frac{db}{dt} = KbC^n \qquad (3)$$

After integration

$$\text{Log } \frac{B}{b} = KC^n t \qquad (4)$$

"C" is the concentration of disinfectant and "n" the exponent indicating the order of reaction. This may be determined from 2 experiments in which different values of "C" are used.

$$n = \log_{10} \frac{k'}{k'} \div \log_{10} \frac{c'}{c} \qquad (5)$$

"C" and "n" being known, "K," the velocity constant in Equation 4, may be calculated. The influence of temperature may be mathematically expressed by the equation

$$\frac{K'}{K} = \theta^{(T' - T)} \qquad (6)$$

in which "K'" and "K" are constants of the reaction at temperatures "T" and "T'," respectively, and θ is the temperature coefficient. This coefficient is important since the action of the disinfectant is proportional to the temperature. With the value of θ known, the velocity constant of a disinfectant at any temperature may be calculated from the equation

$$K_{T^o} = K_{20^o} \times \theta^{T^o - T^{20^o})} \qquad (7)$$

These three constants, the velocity constant "K," the concentration exponent "n," and the temperature coefficient θ, are supposed to define the three essential characteristics of any disinfectant. These three values may be obtained for a given antiseptic substance and compared with the same values obtained for carbolic acid.

While such methods as the two described may have some advantages, they certainly have at present many disadvantages. But they serve to illustrate the hope that an intensive study of the physical chemistry of disinfection may not be without practical importance. In the investigation to be described in this and following papers we have undertaken to apply physico-chemical principles and methods to what may be considered a biologic problem; that is, the mechanism of disinfection.

The Action of the Hydrogen Ion

The first work on the relation of electrolytic dissociation to the destructive action of toxic substances was done by Dreser. Later, other investigators experimented with different metallic salts.

Bial,[5] Chick,[3] Madsen and Nyman,[2] and Reichel[6] found in the case of mercury salts that the disinfecting action was proportional to the Hg-ion content, and was decreased on the addition of sodium chlorid. Other metallic salts behaved in a similar way.

Acids show a like behavior, their antiseptic action depending, at least partly, on the degree of dissociation, and on the concentration of the hydrogen ion. For example, Bial[5] found that the less an acid was dissociated, the higher the concentration required for a given killing power toward yeast. In the presence of a salt having a common ion, a condition which gives a decrease in the hydrogen-ion concentration, this power is much diminished.

Other investigators have observed the same phenomenon. Winslow and Lockridge,[7] using B. coli and B. typhosus, found that the disinfecting power of acids was proportional to the concentration of the hydogen ion, but that organic acids have a stronger action than inorganic acids having the same hydrogen-ion concentration. That the anion has little disinfecting power has been shown by Daniels[8] in some experiments with alkali formates.

In some recent papers Paul, Birstein, and Reuss[9] put forward the view that not only does the hydrogen-ion concentration play an important rôle in the kinetics of disinfection, but that the anions and the undissociated molecules are also responsible factors; that is, they accelerate the action of the hydrogen ions. These views are supported by considerable experimental evidence, but we can hardly regard it as conclusive, particularly in view of some preliminary experiments of our own.

The first part of this investigation has therefore been undertaken in order to show, conclusively, the rôles which undissociated acids, hydrogen ions, and anions play in disinfection. For the representative acid we have chosen formic acid, because of its relatively high germicidal power and because its physico-chemical constants either are known or can be readily determined. It is also well adapted for experiments on adsorption, which form a part of this investigation.

Test Organism.—The organism used for the investigation was B. typhosus (Hopkins), obtained through the courtesy of the Hygienic Laboratory, Washington. From the stock culture agar slants were made, kept in an ice chest, and transferred about once a month. The culture actually used was made by inoculating nutrient broth (+ 1.5), and making transfers into fresh broth tubes twice again, the period between each transfer being 24 hours. The growth in the third tube was used after exactly 24 hours. In this way the resistance of the organism was kept practically constant. If more than 3 transfers into broth were made, the culture gradually lost its vitality. Table 1, which it seems worth while to insert here, will emphasize this statement.

[5] Ztschr. f. physical. Chem., 1902, 40, p. 513.
[6] Biochem. Ztschr., 1909, 22, p. 149.
[7] Jour. Infect. Dis., 1906, 3, p. 547.
[8] Thesis, Mass. Inst. Technology, 1912.
[9] Biochem. Ztschr., 1910, 29, pp. 202, 239.

TABLE 1

THE RELATION BETWEEN AGE AND THE RESISTANCE OF BROTH CULTURES OF B. TYPHOSUS
TOWARD FORMIC ACID

Dilution of HCOOH	Time in Minutes Required for Complete Killing		
	Transferred for 3 Days	Transferred for 20-25 Days	Transferred for 3 Months
1-200..	25	25	5
1-300..	35	45	10
1-400..	50	55	12½
1-500..	105	95	12½
1-600..	175	130	15

Temperature.—All our experiments were performed at 20 C. This temperature was maintained in a water bath by means of an automatic electric heater and regulator, and did not vary more than 0.05° during any experiments, except those requiring long periods of time, in which the variation was slightly greater, but well within the limits of error of the work.

Medium.—The medium used was nutrient broth, made from Liebig's Extract, with a reaction of + 1.5 (phenolphthalein). In order to have the culture tubes all contain exactly the same amount of broth, a Vanier automatic 10-c.c. pipet was used for filling them. This proved an accurate, easy, and rapid method.

Method.—In all the tests recorded in this paper, the technic used was substantially that proposed by the Hygienic Laboratory for the standardization of disinfectants,[10] the time of exposure being extended, of course, to suit our needs. The broth culture of B. typhosus (Hopkins) as described above, was filtered through sterile filter paper and placed in the water bath. After it had remained there long enough to come to the required temperature, a series of seeding tubes containing 5 c.c. of various amounts of acid or acid-salt mixture were inoculated with 0.1 c.c. of the filtered culture (approximately 9,000,000 bacteria) from a 1-c.c. graduated pipet at ½-minute intervals. Subcultures into broth were then made with the standard loop (4 mm. in diameter) after the required time had elapsed. The subculture tubes were incubated at 37 C. for 48 hours and were then examined for growth. We found in all cases but one that 48 hours were sufficient for developing any surviving organisms.

EXPERIMENTAL RESULTS

The first tests were made to determine the relation of the concentration of hydrogen ion, formed by the dissociation of formic acid in water solution, to its disinfecting power. Table 2 gives the data and Chart 1 shows a curve obtained by plotting the time of exposure required for complete disinfection against the hydrogen-ion concentration. Similar curves could be obtained for the formate anion or the undissociated molecule. The disinfecting power might therefore be proportional to any one of the three.

[10] Bull. Hyg. Lab., U. S. P. H. and M.-H. S., 1912, 82.
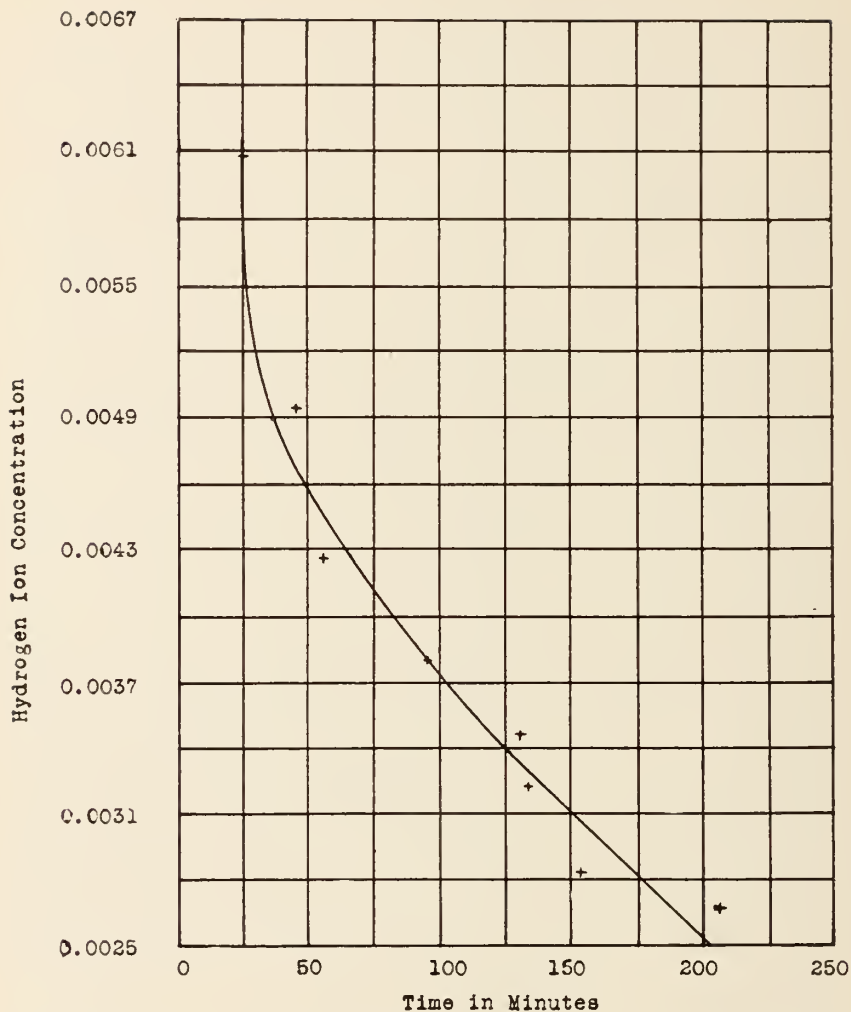
Chart 1.—The relation of the concentration of hydrogen ion, formed by the dissociation of formic acid in water solution, to its disinfecting power.

If the disinfecting action is proportional to the hydrogen-ion concentration, then by decreasing the latter in an acid solution of a given normality, the disinfecting action should be correspondingly decreased. The simplest method of changing the hydrogen-ion concentration is by adding a salt with a common anion. For this purpose ammonium formate and sodium formate were separately added to 0.0723 normal formic acid. In each experiment controls were made with pure acid and

with pure salt.   No appreciable disinfecting power was shown by the
latter.   Tables 3 and 4 show the results.

TABLE 2

Relation of Concentration of Formic Acid to Disinfection

Dilution by Volume	Normality	H⁺ Ion Concentration, Mols per Liter	Time in Minutes Required for Complete Killing
1-200	.1879	.006072	25
1-300	.1253	.004944	45
1-400	.09393	.004260	55
1-500	.07514	.003796	95
1-600	.06262	.003450	130
1-700	.05367	.003210	135
1-900	.04174	.002818	155
1-1000	.03757	.002670	205

Culture transferred 20-25 days.
H⁺ ion concentration calculated on basis[11] of $K_{20}° = 2.04 \times 10^{-4}$

TABLE 3

The Effect on Disinfection of the Addition of Ammonium Formate to Formic Acid

Composition of Disinfectant		Time in Hours for Complete Killing
.0723 HCOOH + 0.0 N	HCOONH₄.............................	2¾
.0723 HCOOH + 1.0 N	HCOONH₄.............................	Not complete in 8 hours
.0723 HCOOH + 0.6 N	HCOONH₄.............................	Not complete in 8 hours
.0723 HCOOH + 0.2 N	HCOONH₄.............................	Not complete in 8 hours
.0723 HCOOH + 0.08 N	HCOONH₄.............................	7
.0723 HCOOH + 0.04 N	HCOONH₄.............................	5¼
.0723 HCOOH + 0.02 N	HCOONH₄.............................	4
.0723 HCOOH + 0.004 N	HCOONH₄.............................	2½
.0723 HCOOH + 0.0015 N	HCOONH₄.............................	2¼
.0 N HCOOH + 2.0 N	HCOONH₄.............................	Not complete in 8 hours
.0 N HCOOH + 1.0 N	HCOONH₄.............................	Not complete in 8 hours
.0 N HCOOH + 0.08 N	HCOONH₄.............................	Not complete in 8 hours

Culture transferred 3 days.

TABLE 4

The Effect on Disinfection of the Addition of Sodium Formate to Formic Acid

Acid-Salt Mixture				Pure Acid	
Normality Acid	Normality Salt	H⁺ Ion Concentration, Mols per Liter	Time in Minutes for Killing	H⁺ Ion Concentration, Mols per Liter	Time in Minutes for Killing
.1879	.006515	.00577	10	.006072	5
.1253	.004343	.00475	15	.004944	10
.09393	.003317	.00411	15	.004260	10
.07514	.002605	.00337	20	.003796	12½
.06262	.002171	.00337	25	.003450	15

Culture transferred 2½ months.
    The hydrogen-ion concentrations for the acid-salt mixtures were calculated on the
assumption of complete ionization of the salt.
    Ratio of HCOOH/HCOONa = 29.

[11] H. Jahn: Ztschr. f. physical. Chem., 1895, 16, p. 85.

Tables 3 and 4 give typical data, which may be easily duplicated. They show clearly certain tendencies:

1. The addition to an acid of a salt containing an anion common to the acid greatly decreases the acid's disinfecting power, except when only very small amounts of the salt are added.

2. The decrease in disinfecting action is only approximately proportional to the change in hydrogen-ion concentration . In other words, the retarding action of the salt is greater than can be accounted for by the change in ionization produced.

3. The formate ion has, of itself, no antiseptic properties.

4. The undissociated formic acid molecule has little, if any, disinfecting action, since an increase in its concentration, obtained by adding salt to acid, did not cause increased activity of the solution.

5. The hydrogen ion is the agent responsible for disinfecting power. This, however, may be influenced by the presence of the formate ion or of undissociated formic acid molecules.

The results obtained, which show decreased disinfection in the presence of salts, are in direct contradiction to the observations of Bial,[5] who found that the addition of a neutral salt with a common ion to an acid, first decreased its antiseptic power but increased it on further additions of the salt. For example, in an acetic acid solution of 0.025 normality the antiseptic power was decreased by the addition of sodium acetate in low concentrations, but when a concentration of 1.2 normal was reached its power was greatly increased.

Our conclusion that the hydrogen ion is the responsible agent may be further tested by the use of an equation proposed by Chick.[3] She found that a logarithmic relation existed between the concentration of a disinfectant like phenol and the time taken for disinfection. That is, the expression $1/t_n\text{-}t_0 \log C_n t_n/C_0 t_0$ remained constant in value. With silver nitrate and B. paratyphosus the equation also held, since silver nitrate in dilute solution is practically all dissociated. The same was true of mercuric chlorid, if in place of concentration of $HgCl_2$ numbers were inserted representing the concentration of mercuric ion. These facts confirm the theory that the metallic ion is the true disinfecting agent in the case of metallic salts. Hence, in our case, if the hydrogen ion is the real disinfecting agent, this same equation should give constant values when calculated on the basis of the ion concentration. Table 5 gives the values calculated with both the total concentration of the acid and that of the hydrogen ion.

TABLE 5

Constants Calculated for Total Acid and for Hydrogen Ion

Normality	H+ Ion Concentration, Mols per Liter	Time in Minutes for Killing	Values of Expression $\dfrac{1}{C_0-C_n}\log_{10}\dfrac{C_n t_n}{C_0 t_0}$	
				K_h
.1879	.006072	25	....	.....
.1253	.004944	45	1.27	147.3
.09393	.004260	55	0.44	104.0
.07514	.003796	95	1.61	165.1
.06262	.003450	130	1.91	179.5
.05367	.003210	135	1.40	159.2
.04174	.002818	155	1.70	141.0
.03757	.002670	205	1.43	163.8

K_n = constant calculated from normality with initial values C_0 = 0.1879 and t_0 = 25 min.
K_h = constant calculated from H+ ion concentration with initial values C_0 = 0.006072 and t_0 = 25 min.

While these results are not absolutely definite, still the percentage deviation in K is less when the calculation is made on the basis of the hydrogen-ion concentration. This therefore aids in confirming our experimental results.

If the hydrogen ion is the true disinfecting agent, then we must account for the fact that when its concentration is decreased by adding a common-ion salt its disinfecting power is more diminished than can be accounted for by its change in concentration. Furthermore, Bial,[5] Frei and Margadant,[12] Scheurlen,[6] and Eisenberg and Okolska[13] have all demonstrated that neutral salts which themselves are not disinfectants or antiseptics have the power of improving the quality of a disinfectant to which they are added. For instance, small concentrations of salt will considerably increase the poisonous action of cresol, and a 0.1% phenol solution containing just enough sodium chlorid not to cause turbidity, is as efficient a disinfectant as a concentrated phenol solution.

Why, then, should a formate have apparently just the opposite action when added to a formic acid solution? In order to answer this and to throw more light on the real retarding agent, another series of experiments was made. If we could add a formate to a solution of formic acid in such a way that the concentrations of hydrogen ion and of undissociated formic acid would not be affected, we should be able to show the true action of the salt. This may be done by the use of the so called "Isohydric Principle."

[12] Ztschr. f. Infektionskrankh., 1914, 15, pp. 273, 350, 407.
[13] Centralbl. f. Bakteriol., I, O., 1913, 69, p. 312.

In general, when we mix two electrolytic solutions, each dissolved substance alters the number of ions and thus affects the dissociation of the other. From the law of mass action, however, as applied to electrolytic equilibrium, we ascertain that there must be certain solutions which can be mixed together without alteration in the nature or number of ions, and therefore without affecting the conducting power, or the dissociation of either of the two dissolved substances. Such solutions are said to be isohydric. The following is the derivation, mathematically, for isohydry in the case of formic acid and ammonium formate solutions. Before mixing, let us denote the concentration of NH_4 ion by C_{NH_4}; of the formate ion of the salt, C_{Fs}; of the formate ion of the acid, C_{Fa}; of H^+ ion, C_H; and of the undissociated acid molecule, C_{HF}. After mixing, they are denoted respectively by C'_{NH_4}, C'_F, C'_H and C'_{HF}. "v_s" equals the volume of the salt solution before mixing and "v_a" equals the volume of the acid solution before mixing. The total volume after mixing is then $v_s + v_a$.

$$C'_H = \frac{C_H \cdot v_a}{v_a + v_s}$$

$$C'_F = \frac{C_{Fa} \cdot v_a}{v_a + v_s} + \frac{C_{Fs} \cdot v_s}{v_a + v_s}$$

$$C'_{HF} = \frac{C_{HF} \cdot v_a}{v_a + v_s}$$

The equilibrium equation for the acid before mixing is

$$\frac{(H^+)\,(F^-)}{(HF)} = K_1 \text{ or } \frac{C_H \cdot C_{Fa}}{C_{HF}} = K_1$$

Its equilibrium equation after mixing will be

$$\frac{C'_H \, C'_F}{C'_{HF}} = K_2$$

After substitution of the values given

$$\frac{C_H \cdot v_a}{v_a + v_s} \left(\frac{C_{Fa} \cdot v_a}{v_a + v_s} + \frac{C_{Fs} \cdot v_s}{v_a + v_s} \right) = K_2 \cdot \frac{C_{HF} \cdot v_a}{v_a + v_s}$$

$$\text{or } \frac{C_H}{C_{HF}} \left(\frac{C_{Fa} \cdot v_a}{v_a + v_s} + \frac{C_{Fs} \cdot v_s}{v_a + v_s} \right) = K_2$$

In order that the ionization of the acid may not be changed on mixing, K_1 must be equal to K_2

Hence

$$\frac{C_H \cdot C_{Fa}}{C_{HF}} = \frac{C_H}{C_{HF}} \left(\frac{C_{Fa} \cdot v_a}{v_a + v_s} + \frac{C_{Fs} \cdot v_s}{v_a + v_s} \right)$$

$$C_{Fa} = \frac{C_{Fa} \cdot v_a}{v_a + v_s} + \frac{C_{Fs} \cdot v_s}{v_a + v_s}$$

This ($K_1 = K_2$) can only be true when

$$C_{Fa} = C_{Fs}$$

For then

$$C_{Fa} = \frac{C_{Fa} \cdot v_a}{v_a + v_s} + \frac{C_{Fa} \cdot v_s}{v_a + v_s}$$

$$= C_{Fa}\left(\frac{v_a + v_s}{v_a + v_s}\right) = C_{Fa}$$

We may say, therefore, that solutions of electrolytes are isohydric when the concentration of the common ion in the different solutions is the same. In other words, if we make the concentrations of the anions of our solutions of formic acid and ammonium formate equal before mixing, there will be no change in the ionization of the acid after mixing. Experiments were tried with 5 such solutions to determine the effect of the salt on the disinfecting power of the acid.

The procedure for these isohydric experiments was as follows:

1. Calculate C_{F_a} in 20 c.c. of x normal formic acid solution.

2. Calculate the volume of x′ normal ammonium formate solution that contains the same concentration of anion.

3. Mix the two electrolytes.

4. Calculate C_H in the mixture.

5. (a) Take 5 c.c. of this mixture for the experiment.

(b) Take 5 c.c. of pure acid containing the same H⁺ ion concentration as in 4 and run an experiment simultaneously with (a).

The concentrations of the formate ion from the salt were obtained by using the average γ values of univalent salts taken from Noyes and Sherrill's "General Principles of Chemistry."

TABLE 6

IONIZATION OF AMMONIUM FORMATE SOLUTIONS

Concentration of HCOO⁻ from HCOONH₄ Mols per Liter	Normal Concentration of Salt	Values of γ for Univalent Salts
.00096	.001	.96
.0092	.01	.92
.0180	.02	.90
.0435	.05	.87
.0840	.10	.84

TABLE 7

CONCENTRATIONS OF DIFFERENT COMPONENTS IN THE ISOHYDRIC MIXTURES

Iso-hydric Mix-ture	Volume of).03757 N HCOOH	Volume of 0.0095 N HCOONH₄	Volume of Mix-ture	Concentration in Mixture of			Normality of Acid Blanks with Same a Concentration as Mixture
				H⁺ ion	NH⁺₄ ion	HCOO⁻ ion	
1	20	8	28	.00618	.00247	.00865	.194
2	20	12	32	.00541	.00324	.00865	.150
3	15	12	27	.00481	.00384	.00865	.119
4	15	15	30	.00433	.00432	.00865	.0968
5	10	14	24	.00360	.00505	.00865	.068

•

TABLE 8

Isohydric Experiments I and II with Ammonium Formate

	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	II	II	II
Minutes	5	7½	7½	7½	10	10	10	12½	15	15	20	20	25	25	30	30	35	32½	40	35	45	37½		40						55	60	65	70
Mixture 1	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	:	−	−	−	−	:	−						−	−	−	
Blank 1	:	:	:	:	:	:	:	.	:	:	+	+	+	−	+	−	+	−	−	−			:										
Minutes	7½	12½	10	15	15	15	15	17½	20	20	25	22½	30	25	35	30	40	35	45	40	50	45	50	50	55	55	60		60	55	60	65	70
Mixture 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	:	−	−	−	+	−	−	:	−	−	−	−	−		−	−	−	−	−
Blank 2	+	:	+	:	+	:	+	:	+	+	+	+	+	+	+	:	:	:	:	:	:	:	−	:	:	:	:		:	−	−	−	−
Minutes	10	12½	15	15	20	15	20	17½	20	22½	35	20	35	25	55	30	60	35	65	40	70	45	90	50		55	90		140	55	60	65	70
Mixture 3	+	+	+	+	+	+	+	+	:	:	:	+	:	+	:	+	:	+	:	+	:	+	+	−		+	+		−	−	−	−	−
Blank 3	:	:	:	:	:	:	:	:	+	+	+	+	+	+	+	+	+	−	+	−	:	−	:	−		:	:		−	−	−	−	−
Minutes	30	20	35	25	40	25	55	40	80	35	90	95	100	100	110	105	120	110	130	115		120	130	115	50	120	150		140	120	125	130	135
Mixture 4	+	+	+	−	−	−	+	−	+	−	+	+	+	:	−	−	+	+	−	−		−	−	−	−	−	−		−	−	−	−	−
Blank 4	:	:	:	:	:	:	:	:	:	:	+	+	+	+	+	−	+	+	−	−		−	−	−	−	−	−		−	−	−	−	−
Minutes	100	35	110	40	120	40	180	55	180	60	190	60	200	160	210	170	120	175		180		185		180	50	190				185	190	195	200
Mixture 5	−	+	−	+	−	+	+	:	+	:	−	:	−	+	−	+	−	−		−		−		−	−	−				−	−	−	−
Blank 5	:	:	:	:	:	:	+	:	+	:		:		+		+		−		−		−		−	:	−				−	−	−	−

The time in minutes gives the exposure of 0.1 c.c. of broth culture of B. typhosus (Hopkins). The sign + signifies growth; − signifies complete killing.

Culture transferred 3 days.

TABLE 9

ISOHYDRIC EXPERIMENT 3 WITH SODIUM FORMATE

Minutes	5	7½	10	15	20	25	30	35	40	45
Mixture 1	+	+	+	—	—	—	—			
Blank 1	..	..	..	..	+	—	—	—	—	—

Minutes	7½	10	15	20	25	30	35	40	45
Mixture 2	+	+	—	—	—	—			
Blank 2	..	..	..	+	+	—	—	—	—

Minutes	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
Mixture 3	+	—	+	+	—	—									
Blank 3	..	..	..	..	+	—	+	—	+	+	+	+	+	—	—

Minutes	10	15	20	25	30	35	40	100	105	110	115	120	125	130	135	135
Mixture 4	+	+	—	—	—	—	—									
Blank 4	..	..	..	..	..	..	..	+	+	+	—	—	—	—	—	

Minutes	35	40	45	50	55	60	65	70	75	150	155	160	165	170	175	180
Mixture 5	+	+	+	+	—	—	—	—	—							
Blank 5	..	..	..	..	..	..	..	..	..	+	+	+	—	+	—	—

See notes with Table 8.

Tables 8 and 9 show the increase in disinfecting power caused by the addition of formates to formic acid. As no change was made in the concentration of the hydrogen ion or of the undissociated formic acid molecule, the increase can be attributed only to the presence of increased amounts of the formate ions. Since these have been shown to have no direct disinfecting action, they must therefore act as accelerating or catalyzing agents. The retarding effect noted in Tables 3 and 4 must therefore be due to the decrease in hydrogen-ion concentration and probably also to a negative catalytic action of the undissociated formic acid. In any acid solution the presence of anions, formed by dissociation of the acid, will cause an acceleration in the disinfecting action, dependent on the nature of the anions, and there will also be a retarding effect due to the presence of the undissociated molecules. The apparent antiseptic power of the acid will be a combination of the power of the hydrogen ion with these two influences.

As a large amount of work has been done on the action of salts as accelerators in disinfection, it is advisable to discuss the results recorded in the three last tables in the light of results obtained where there is no common-ion effect.

THE ACTION OF SALTS

For our purposes, salts may be divided into 2 classes—those which are themselves disinfectants, such as silver nitrate and mercuric chlorid; and those which have no such properties, such as sodium

chlorid, sodium nitrate, etc. For those of the first class the disinfecting action has been shown to be proportional to the concentration of the cation.[4] This is analogous to the case of acids, which we have discussed. The second class is of particular interest in a study of disinfection.

Bial[5] in 1902 observed that the addition of a salt, with a common ion, to an acid influenced its antiseptic power. Frei and Margadant[12] found that salts in small quantities greatly influenced the disinfecting action of cresol, but that the effect was not proportional to the concentration of the salt, and that the action of the salt and cresol was not additive. They also suggested that the ions from the salts influenced the disinfectant in opposite directions, the final effect being the resultant of the two actions. Scheurlen[6] found that sodium chlorid increased the efficiency of phenol. Eisenberg and Okolska[13] showed that lipoid-soluble antiseptics were greatly improved by the addition of neutral salt solutions. In almost all cases investigated, neutral salts were found to increase disinfecting properties. Exceptions were noted in the cases[13] of mercuric chlorid, urea, and potassium permanganate, in which addition of salt retarded the action. Alkaline salts, such as K_2HPO_4, $KAsO_2$, etc., acted more energetically than did neutral salts.

That some relation exists between the concentration of an added salt and its accelerating effect, is quite evident. Reichel[6] and Paul, Birstein, and Reuss[9] have made experiments and suggestions along this line. The last named suggested the equation

$$K_2 = K_1An$$

in which "A" is the accelerating factor of the salt, "n" its concentration, and "K_1" and "K_2" the disinfection-velocity constants of the acid and of the acid-salt mixture, respectively. The relation

$$K_2 = K_1 (1 - An)$$

has also been suggested, in which "A" is a proportionality factor.

The action of the various anions and cations has also been studied by Frei and Margadant,[12] who arranged each in a series showing the extent of its influence. In general, the anions have a much stronger action than have the cations. The latter sometimes retard the disinfecting action.

In order to know definitely whether or not salts other than formates have the same influence on the disinfecting power of formic acid as on other disinfectants, tests were made with sodium chlorid and sodium nitrate. The results are recorded in Tables 10 and 11.

These results confirm those of other investigators. We wish to point out particularly the very great effect produced by very small amounts of salt. The influence on the disinfecting power of formic acid is essentially of the same order as that of sodium formate and

ammonium formate in isohydric mixtures. The salts by themselves show no appreciable antiseptic action. A series of experiments was also made with higher concentrations of sodium nitrate—0.484, 0.601, 0.683, 0.742 and 0.819 normal, respectively—and the same acid concentrations as shown in the tables, but the action was so rapid that the time of killing could not be determined.

TABLE 10

The Influence of a Neutral Salt on the Disinfecting Power of an Acid

Formic Acid and Sodium Nitrate

Minutes	5	10	15	20	25	30	35
Mixture 1	+	−	−	−			
Blank 1	..	+	+	−	−	+	−

Minutes	7½	10	15	20	25	30	35	40
Mixture 2	+	+	−	−				
Blank 2	..	..	+	+	−	−	−	−

Minutes	10	15	20	25	30	35	50	55	60	65	70	75	80
Mixture 3	+	+	+	−	+	−							
Blank 3	..	..	..	..	..	..	+	+	−	−	−	−	−

Minutes	10	15	20	25	30	35	40	100	105	110	115
Mixture 4	+	+	−	+	−	−	−				
Blank 4	..	..	..	..	..	..		−	−	−	−

Minutes	20	25	30	35	40	45	155	160	165	170
Mixture 5	+	+	+	−	−	−				
Blank 5	..	..	..	..	..	..	+	−	−	−

The salt concentrations in the mixtures are, respectively, 0.00271, 0.003563, 0.00422, 0.00475, 0.00554 normal.

The acid concentrations, designated as blanks, are the same as those of the isohydric mixtures (See Table 7).

TABLE 11

The Influence of a Neutral Salt on the Disinfecting Power of an Acid

Formic Acid and Sodium Chlorid

Minutes	10	15	20	25	30	35
Mixture 1	+	+	+	−		
Blank 1	+	+	+	−	−	−

Minutes	7½	10	15	20	25	30	35	40
Mixture 2	+	+	+	−	−			
Blank 2	..	..	+	+	+	+	+	−

Minutes	7½	10	15	20	25	30	35	65	70	75	80
Mixture 3	+	+	+	−	−	−					
Blank 3	..	..	..	..	..	..	..	+	+	+	−

Minutes	20	25	30	35	40	45	100	105	110	115	120	125
Mixture 4	+	+	+	+	−	−						
Blank 4	..	..	..	..	..	..	+	+	+	−	+	−

Minutes	50	55	60	65	70	75	140	145	150	155	160	165	170
Mixture 5	+	+	+	−	+	−							
Blank 5	..	..	..	..	..	..	+	+	+	−	−	+	−

See notes with Table 10.

SUMMARY

Acids act as disinfectants through the agency of the hydrogen ions produced by electrolytic dissociation.

The disinfecting power of an acid is approximately proportional to the hydrogen-ion concentration.

The addition to an acid of a salt containing an anion common to this acid, diminishes its disinfecting power, as the result of a decrease in the hydrogen-ion concentration and an increase in the concentration of the undissociated acid molecules.

Salts which do not appreciably affect the dissociation of an acid, greatly increase the disinfecting properties of the acid.

Acid anions are positive catalyzers and undissociated acid molecules are negative catalyzers in acid disinfection.

A discussion of the mechanism of the salt effect is so closely connected with that of the mechanism of disinfection that it will be left for a subsequent article, based on work already completed in this laboratory and on investigations at present under way, involving an intensive application of physical chemistry to the problem of disinfection.

THE DIPHTHEROID BACILLUS OF PREISZ-NOCARD FROM EQUINE, BOVINE, AND OVINE ABSCESSES *

ULCERATIVE LYMPHANGITIS AND CASEOUS LYMPHADENITIS

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With Fisher[1] one of us (H.) has described a series of abscesses in 11 horses and 1 calf from all but one of which we obtained pure primary cultures of a diphtheroid bacillus identical with an organism recovered by us from caseous lymphadenitis in sheep. This organism appears to be the same as that described by certain American and European writers as the bacillus of Preisz-Nocard, which causes a disease among horses generally known as ulcerative lymphangitis. There is some question as to whether all the cases we observed were truly lymphangitic; at any rate one was very suggestive of it, and the deep origin of many of the abscesses seemed to indicate a localization in certain lymphatic nodes. We wish now to describe some of the bacteriologic aspects of our work, which is the first to show the occurrence of equine and bovine infections due to B. Preisz-Nocard in the United States.

MORPHOLOGY

Smears from the pus of the abscesses were stained by Ziehl-Nielsen's carbolfuchsin, followed by 10% HCl for acid-fast organisms, and by Gram's method. Gram's stain showed in each case typical gram-positive cocco-, diplo-, or diphtheroid bacilli of pleomorphic character, frequently in palisade arrangement, at other times in irregular masses.

The pus, diluted in glucose broth, was streaked out therefrom on rabbit- or horse-blood agar plates and tubes. Gram stains were made of cultures incubated overnight at 37 C., to ascertain the purity of the original culture. However, isolated colonies were always picked up for further study, and after confirming the positive reaction to Gram's stain and the non-acidfastness in 24-hour cultures on blood agar, the various morphologic and physiologic properties of the bacillus were determined.

Plain agar and glycerin agar proved unsuitable for continued cultivation. On agar containing 7.5% rabbit or horse blood growth is moderate after 24 hours at 37 C., freshly isolated cultures growing less vigorously than older ones. Isolated colonies are circular, umbonate, ochraceous or cretaceous, and opaque. On incubation for several days they may reach a size of 8 to 10 mm., and show formation of concentric rings about the elevated papilliform center.

When touched with the needle the growth is peculiarly friable, the colony breaking apart readily. Growth is best at 37 C., but longer incubation at 20 C. in the dark is successful. Hemolysis of plated blood agar is marked in the absence of fermentable carbohydrates.

On Löffler's blood serum, an excellent medium, for which equine or bovine serum serves equally well, growth is similar to that on blood agar, with more suggestion of a yellowish insoluble pigment on Löffler's blood serum, particularly that of bovine origin. (Our blood serum consisted of 3 parts horse serum with 1 part beef broth containing 1% Witte's peptone, 0.5% NaCl, 1% dextrose, and 5% glycerin, the mixture being filtered through a Berkefeld filter, tubed, slanted, and sterilized in the autoclave at 10 pounds pressure for 30 minutes. Several strains of B. diphtheriae grown upon media prepared in this way and on media prepared by the usual fractional sterilization in the Arnold have shown no difference in growth or morphology.) On Löffler's blood serum the organism is diphtheroid, with a tendency, as with B. diphtheriae, tho less marked, toward slender, clubbed forms showing metachromatic granules with Löffler's methylene blue. On this medium the length was from 1.0 to 1.6 microns, and the width, from 0.4 to 0.5 micron.

Gelatin at 37 C. affords a moderate granular growth, but when placed in the ice-chest for an hour or so or at 20 C. it becomes hard. Tests were made daily for liquefaction for 5 days.

One-percent glucose broth yields a scanty granular deposit with slight or no pellicle-formation or clouding of medium. Broth containing no glucose develops a pellicle similar to that of B. diphtheriae with analogous sinking and formation of granular sediment. The elaboration of a soluble toxin and hemolysin in this medium will be discussed later.

Anaerobic growth in dextrose broth under hydrocarbon oil proceeds as without oil; these organisms are therefore facultative anaerobes, but shake cultures in deep dextrose agar showed their preference for aerobic conditions. The organisms were lacking in motility. No encapsulated forms were found.

These organisms in their growth on agar containing gentian violet, according to the method of Churchman,[2] were distinctly inhibited by a concentration of 1 : 100,000.

Of significance with relation to Hodgkin's disease in man is the fact that antiformin dissolved cultures of 6 strains of B. Preisz-Nocard, whereas the organisms observed by Fraenkel and Much[3] and cultivated by Bunting and Yates[4] and others from this disease, tho non-acidfast, are said to be frankly resistant to antiformin.

Cultures 10 days old on blood agar were suspended each in 10 c.c. of 0.85% NaCl, equal parts of antiformin added, and the mixtures left at 36 C. for 2 hours. They were then centrifugated, the supernatant fluid decanted, refilled with salt solution, again centrifugated and again the clear fluid decanted. Slides stained by the Ziehl-Nielsen and Gram methods failed to show anything but debris, while plants on blood agar remained barren. Untreated emulsions, however, showed the usual gram-positive cocco-bacilli and cultures on blood

[2] Jour. Exper. Med., 1912, 16, p. 221.
[3] Ztschr. f. Hyg. u. Infektionskrankh., 1910, 67, p. 159.
[4] Arch. Int. Med., 1913, 12, p. 236.

agar were successful. The experiment was repeated with a mixture of cultures 3 days old, with similar results at the end of 1 hour's treatment.

From these experiments it would seem that the organism in Hodgkin's disease is dissimilar.

Fermentation Reactions.—These reactions, tho often irregular in freshly isolated cultures, necessitated provisional identification by observation of growth on solid media, morphology, and tinctorial reactions. Each of our cultures was acclimatized to blood agar by weekly transplantation and, after 24 hours' incubation at 37 C., maintenance in diffuse light at about 20 C. A few transfers sufficed to render the reactions in Hiss's serum water media (carbohydrate 1%) constant. Dextrose (Eimer and Amend), levulose (Kahlbaum), and maltose (Merck), were fermented with formation of acid; with dextrose and maltose coagulation occurred earlier for all strains than with levulose. No gas-formation was found. Lactose (Eimer and Amend), saccharose (Eimer and Amend), raffinose (Eimer and Amend), dextrin (Merck), inulin (Eimer and Amend), glycerol (Braun-Knecht-Heimann), mannite (Eimer and Amend), and dulcite (Eimer and Amend), were not acidified in 5 days at 37 C. Litmus milk was unchanged in appearance.

These media were specially prepared, care being taken to prevent hydrolysis by overheating. Subplants were made from each tube, after 24 hours at 37 C., on blood agar to confirm the success of implantation and freedom from contamination. We emphasize this step in technic as a check against error in fermentation tests; for example, failure of proliferation, or extraneous organisms.

Table 1 shows that B. Preisz-Nocard in its fermentation reactions most resembles B. flavidus. The former may be differentiated by its failure to produce acid in glycerol.

TABLE 1

FERMENTATION REACTIONS OF DIPHTHEROID BACILLI

	Dextrose	Maltose	Glycerol	Saccharose	Dextrin	Mannite
Urethral diphtheroids (9)............	+	+	..	+	+	—
B. diphtheriae (5) (6) (7) (8) (9)....	+	+	+	—	+	—
B. xerosis (5) (6) (7) (8) (9).....	+	+	±	+	—	—
B. Hoagii (8)........	+	—	—	+	—	—
B. flavidus (8)	+	+	+	—	—	. —
B. Preisz-Noeard	+	+	—	—	—	—
B. coryzae segmentosus (9)..........	+	—	—	—	—	—
B. Hofmanni (5) (6) (7) (8) (9).....	—	—	—	—	—	—

The sign + means acid formation; — means no acid formation; the figures following the names of the organisms refer to references in footnotes.

Dunham's peptone salt solution gave a negative nitroso-indol reaction with concentrated H_2SO_4, and no free indol with $NaNO_2$. Nitrate broth, while allow-

5 Jour. Med. Research, 1904, 7, p. 475.
6 Jour. Hyg., 1906, 6, p. 286.
7 Jour. Med. Research, 1907, 17, p. 277.
8 Jour. Infect. Dis., 1912, 11, p. 253.
9 Jour. Path. and Bacteriol., 1913, 18, p. 75.

ing faint development, showed no reduction to nitrites at the end of 5 days' incubation with the usual test reagents.[10]

Bacteriologic observations, confirmed several times on all our strains, showed that the organisms recovered from the horses, ewes and the calf were identical. Such minor variations in intensity as occurred were not referable to differences in host. For comparison we studied a strain of B. pseudotuberculosis-ovis supplied by the American Museum of Natural History. It differed markedly from ours in the formation of an orange pigment, a moist, non-friable growth, vigorous reduction of nitrates to nitrites, and non-pathogenicity for guinea-pigs. We have placed in the American Museum of Natural History cultures from our Cases IV, V, and XII for permanent reference.

Serum Tests.—Agglutination tests failed because of the rapid, spontaneous agglutination of the bacilli in 0.85% NaCl solution. Sera secured from infected horses, as well as from artificially immunized rabbits, were tried.

Hemolysis of Blood Agar Plates.—All our strains destroyed the opacity of agar plates containing 7.5% fresh, defibrinated rabbit or horse blood. Fermentable carbohydrates in the agar inhibited the lysis, as first shown by Ruediger[11] for streptococci and other organisms, and gave rise to a green pigmentation in and about the colony. This reminded us at first of sulfohemoglobin, as mentioned by Abderhalden,[12] but tests of several strains for liberation of sulfids by the method of Darling[13] resulted negatively, altho growth occurred in the first subplant from blood agar on plain agar containing 1% bismuth subnitrate. Incidentally, we confirmed the blackening of this medium by several organisms, particularly B. coli and B. proteus.

It is well known that fermentable carbohydrates in culture media protect the protein from attack and that the alkalinity of cultures is usually due to the liberation of ammonia from split protein, so that we now believe the lysis of corpuscles by certain bacteria to depend on the formation of alkali, which may determine the action of a hemolytic enzyme. But the idea that alkali alone may be responsible is supported by the fact that a drop of $N/20$ NaOH or NH_4OH upon a blood agar plate produces a similar hemolysis, while a drop of $N/20$ HCl produces green coloration analogous to that obtained by the growth of bacteria on blood agar containing fermentable carbohydrates. The differentiation of certain bacteria on the basis of hemolysis may be no more reliable, therefore, than by the use of carbohydrate media.

We have found supernatant fluid from centrifugated week-old cultures in broth (2% Witte's peptone, 0.5% NaCl, veal infusion, + 1)

[10] Jordan, General Bacteriology, 1908, p. 34.
[11] Jour. Infect. Dis., 1906, 3, p. 663.
[12] Textbook of Physiol. Chem., 1908, p. 501.
[13] Amer. Jour. Pub. Health, 1913, 3, p. 233.

slightly hemolytic for washed guinea-pig corpuscles and this hemolytic property is not destroyed by boiling for 10 minutes, a fact which suggests that for this organism hemolysis may be independent of enzyme-formation. A representative experiment follows.

B. Preisz-Nocard II was inoculated October 6, into 500 c.c. of the aforementioned broth in a 2-liter flask. October 9, a slight pellicle rapidly overspread the surface, and thickened as did that of the control, B. diphtheriae (Park and Williams, 8). October 14, the culture was centrifuged and the supernatant fluid decanted. No preservative was added. Normal, defibrinated guinea-pig blood was diluted 1 : 10 in 0.85% NaCl. From a portion the serum was removed by triple centrifugation and decantation.

A mixture of the washed guinea-pig corpuscles diluted 1:10 (1 c.c.) and the filtrate described, boiled and unboiled, after 16 hours' incubation, gave slight hemolysis. A control mixture of uninoculated broth of the same lot (1c.c.) with washed corpuscles diluted 1 : 10 (1 c.c.), gave no hemolysis.

Some of our attempts to secure a soluble hemolysin have been unsuccessful, tho the strains used had in no case lost their hemolytic action on blood agar plates.

PATHOGENICITY

Nocard and Leclainche[14] state that B. Preisz-Nocard is pathogenic for horse, ass, mule, sheep, goat, dog, rabbit, and guinea-pig. Pigeons and fowls are refractory. Our work has been confined mostly to guinea-pigs. In addition to B. mallei, as causing orchitis in guinea-pigs, Wade[15] mentions B. pyocyaneus, Bonner[16] B. coli communis, and Ramon[17] the bacillus of Malassez and Vignal; therefore, little weight can be attached to orchitis as a diagnostic sign without cultural study of the organism. But the regularity with which orchitis follows intra-peritoneal injection of small amounts of B. Preisz-Nocard cultures is interesting. Table 2 shows the outcome of pathogenicity tests with several strains.

Cultures in tubes of 1% dextrose broth were incubated for 48 hours at 37 C. The granular deposit was shaken thoroughly and 1 c.c. injected into male guinea-pigs, all in the same cage. For the subcutaneous injections, animals of lighter weight were purposely chosen.

The animals comprising this series might be considered in 3 groups, according to their behavior after injection and appearance at autopsy.

[14] Les Maladies Microbiennes, 1903, 11, p. 166.
[15] Jour. Infect. Dis., 1913, 12, p. 7.
[16] Lancet, 1913, 185, p. 996.
[17] Ann. de l'Inst. Pasteur, 1914, 28, p. 585.

Guinea-pigs 97, 99, and 101: Subcutaneous tissues of the abdomen edematous; viscera normal, except for slightly hyperemic suprarenal glands, as in guinea-pigs inoculated with a toxic diphtheria culture.

Guinea-pigs 98, 100, 102, and 104: Diffuse peritonitis with little or no subcutaneous edema; highly inflamed suprarenal glands.

Guinea-pig 103: Extreme systemic intoxication on the first, second, and third days; a reduction of 20% in weight the first week. June 17, weight increased to 550 grams; soft abscess 2 cm. in diameter at site of inoculation. June 20, abscess partially reabsorbed; severe orchitis beginning. June 23, both testicles discharging creamy, caseous pus through scrotal walls. July 24, recovery complete.

TABLE 2

PATHOGENICITY OF B. PREISZ-NOCARD FOR MALE GUINEA-PIGS

Case from Which Culture Was Taken	Subcutaneous Injection					Intraperitoneal Injection				
	Guinea-pig		Orchitis	Death	Culture* at Autopsy	Guinea-pig		Orchitis	Death	Culture† at Autopsy
	Number	Weight in Grams				Number	Weight			
Horse III	97	580	None	30 hr.	None	98	740	Slight	18 hr.	Mesentery only
Horse IV	99	430	None	30 hr.	None	100	550	Slight	18 hr.	Mesentery only
Horse I	101	560	None	40 hr.	Liver abscess only	102	570	Slight	18 hr.	Mesentery only
Ox VII	103	600	Chronic severe	Very sick Recovered		104	630	Slight	40 hr.	Mesentery only

* Heart blood and subcutaneous lymph streaked on blood agar.
† Heart blood, peritoneal fluid, and peritesticular fluid streaked on blood agar.
Heart blood cultures were negative in all cases.

This variety in pathologic changes is better shown in the next series,—injected to determine the minimal lethal dose of Strain III. The results are given in Table 3.

The 24-hour growth of a cooked blood agar slant in a tube providing a slanted surface, 2.5 cm. by 6.5 cm., was removed in 10 c.c. of sterile 0.85% NaCl and ground in a mortar to separate the clumps. Dilutions of the agitated suspension were made so that 1 c.c. contained the amount injected intraperitoneally into the guinea-pigs and correspondingly plated on plain agar.

The large doses resulted in symptoms suggestive of intoxication, followed rapidly by death,—a pathologic picture simulating roughly that of guinea-pigs killed by diphtheria toxin. In these and other cases the suprarenal glands seemed to become hyperemic, particularly when death was due to intoxication rather than to the formation of abscesses. Smaller doses failed to intoxicate, but led to emasculation through, first, periorchitis, and finally, invasion of the testicles themselves.

Following the establishment of drainage from such orchitic abscesses, apparent recovery occurred. These experiments indicate the value of small intraperitoneal doses in demonstrating orchitis due to this organism.

We have also studied the production of orchitis in guinea-pigs injected with small doses of the organisms isolated from sheep, Cases IX and XII.

TABLE 3

Result of Decreasing Intraperitoneal Doses of B. Preisz-Nocard

Amount of Emulsion Injected and Plated c.c.	Colonies on Agar Plate (37 C. for 72 hr.)	Guinea-pig		Orchitis*	Other Symptoms	Death	Subcultures
		Number	Weight in Grams				
1.0	Uncount-able	108	750	Marked	Intoxication	48 hr.	Heart blood — Peritoneum + Testicle —
0.1	About 700 ?	109	570	None	Intoxication	48 hr.	Heart blood — Peritoneum + Vas deferens +
0.01	77	110	400	Developing (3) Severe (7) Ul-cerative for 3 weeks	Intoxication first few days	Fully recov-ered (?), but emasculated	
0.001	0	111	680	M a r k e d (6) Ulcerative for 3 weeks	No intoxica-tion; ab-scess on ankle	Fully recov-ered (?), but emasculated	
0 0001	0	112	510	Developing (8) Marked (10) Chronic for 3.5 months	No intoxica-tion	Never fully recovered Used for other experi-ments	

* Figures indicate day of observation following injection.

Forty-eight-hour dextrose broth cultures were injected intraperitoneally in the dosage indicated in Table 4. In each case the volume injected was 1 c.c.

In this series none of the doses was large enough to cause acute intoxication; all the animals suffered from a chronic course of disease, from which, however, none recovered. This severer outcome of the chronic disease probably was due to adverse temperature conditions that did not obtain with those of the previous series; for certain guinea-pigs, apparently recovered from other experiments, died during the cold weather at this time, B. Preisz-Nocard being recovered in pure culture from their visceral lesions.

Some of the lesions noted in this series deserve special mention.

Thus, the apparently normal appearance of Guinea-pig 168 up to the day of death was probably due to an error in infection such that the inoculum failed to reach the peritoneal cavity, passing only through the abdominal

muscles to lodge beneath the parietal peritoneum. There an extensive cohesion started, which, involving the neighboring viscera, finally caused death by occlusion of the intestine. The abscess was strictly localized, the disease showing no evidence of metastasis in this animal.

Guinea-pig 169, on the other hand, developed numerous metastases throughout the lymphatics, with abscesses in the pectoral muscles, on the spleen, and particularly in the omentum. This is one of the few instances in which we recovered a culture from the heart blood. The testicles were destroyed, the tunica vaginalis containing at autopsy a mass of pus only.

In Guinea-pig 171, death was apparently due to a hemorrhage of the portal vein, which had been weakened by a substernal abscess, caused, according to cultures therefrom, by the aurococcus. The usual lesions in the peritoneum

TABLE 4

PATHOGENICITY FOR GUINEA-PIGS OF B. PREISZ-NOCARD ISOLATED FROM SHEEP

| Case from Which Culture Was Taken | Dose In-jected c.c. | Guinea-pig | | Orchitis* | Other Symptoms | Death | Subcultures |
		Num-ber	Weight in Grams				
IX	0.1	163	530	Severe (3)	Local abscess at site of inoculation. Weight at death 410 grams	16 days	Heart blood − Omentum + Tunica vaginalis +
	0.01	167	670	Slight (14) Marked (21)	....	30 days	Heart blood − Omentum + Testicles +
	0.001	168	350	None	Apparently normal, except for slight local induration at site of inoculation	21 days, suddenly	Heart blood − Peritoneal abscess +
XII	0.1	169	670	Marked (3) Severe (10)	Local induration at site of inoculation	15 days	Pectoral abscess + Heart blood + Testicle + Omentum +
	0.01	170	450	Slight (3) Marked (6)	Local induration at site of inoculation	23 days	Testicle + Heart blood +
	0.001	171	380	Slight (6) Marked (20)	....	23 days	Blood from pleural hemorrhage; Aurococcus only

* Figures indicate day of observation following injection.

were present and the testicles had been destroyed. But these lesions might have been due to the staphylococcus. Only Guinea-pig 167 showed any hyperemia of the suprarenals.

Our experience in producing orchitis with the organism from sheep does not coincide with that of Norgaard and Mohler.[13] These writers noted it but once, yet the duration (8 to 15 days) of the disease and the chronic nature of the visceral lesions in their animals indicate that the dose injected (0.3 c.c. to 0.75 c.c.) was not sufficiently large in all cases to destroy the animals before the orchitis could develop. We

[18] Sixteenth Ann. Rep. Bureau of Animal Industry, 1899, p. 638.

have recently tested Strain XII, now cultivated for over a year on artificial media, by injecting 0.01 c.c. of a 4-day glucose broth culture intraperitoneally into a male guinea-pig. Distinct orchitis developed by the sixth day. Guinea-pig 168 is the only instance in which we have failed to produce orchitis by intraperitoneal injections of small quantities of B. Preisz-Nocard from sheep, and there the reason was quite evident upon autopsy. Moreover, we have found the most prominent visceral lesions in such guinea-pigs to occur, not in the liver, but rather in the omental lymph nodes and the spleen.

Intraperitoneal injection of massive doses of culturally identical diphtheroid bacilli of human origin did not produce orchitis in guinea-pigs.

SOLUBLE TOXIN

The behavior of the guinea-pigs killed with larger amounts of culture was strongly suggestive of the acute intoxication produced by diphtheria toxin, and indeed the occurrence of a similar soluble product has been affirmed by Dassonville.[19] His results were confirmed by Carré and Bigoteau,[20] with whose principal conclusions our few experiments in this direction agree. At first, however, our cultures from Cases I, IV, and VII, failed to produce filtrates toxic for guinea-pigs, even in doses of 10 c.c., altho B. diphtheriae (Park-Williams No. 8), utilized simultaneously as a control under similar conditions, produced a filtrate containing more than 100 M.L.D. per cubic centimeter. Yet excellent surface growth, indistinguishable from that of B. diphtheriae, resulted in each flask; hence, in the light of our later knowledge, we attribute the failure to Berkefeld filtration after addition of tricresol.

Supporting the idea of a soluble toxin is the fact that week-old cultures of B. Preisz-Nocard from Case III in broth (2% Witte's peptone, 0.5% NaCl in veal infusion, + 1) proved fatally toxic in a dose of 1 c.c., but the bacilli washed 4 successive times in 0.85% NaCl or broth failed to induce intoxication in a similar dose. One cubic centimeter of the supernatant fluid of a centrifugated culture, however, while not entirely free from living bacilli, yet certainly containing a fraction only of the number injected in the washed sediment, was found to intoxicate fatally in a manner similar to that of the unaltered culture. Recognizing a weakness in these observations, we repeated this experi-

[19] Bull. de la Soc. centr. de Méd. vétérin, 1907, 84, p. 576. Jour. Comp. Path. and Therap., 1908, 21, p. 181.
[20] Rev. gén. de Méd. vétérin., 1908, 11, p. 127.

ment using Strain II, with the added precaution of Berkefeld filtration of the supernatant fluid, secured by centrifugation as follows:

October 6, a 2-liter flask of 500 c.c. veal broth (2% Witte's peptone, 0.5% NaCl, reaction + 0.5) was inoculated with B. Preisz-Nocard (Case II) and incubated at 37 C. There was a granular deposit for 2 days, giving way on the third to a thin pellicle, which became heavier from the fifth to the eighth day. A flask of the same broth inoculated with B. diphtheriae presented a precisely similar appearance from day to day and yielded, when filtered, a toxin containing from 25 to 50 M.L.D. per cubic centimeter.

October 14, the culture of B. Preisz-Nocard was removed, a part centrifugated, and one portion of the sediment washed 4 successive times in 0.85% NaCl, while another portion was similarly treated in uninoculated broth.

Table 5 gives the results of the experiment demonstrating a soluble toxin.

TABLE 5

RESULTS OF EXPERIMENT DEMONSTRATING A SOLUBLE TOXIN IN CULTURES OF
B. PREISZ-NOCARD

Material Inoculated	Guinea-pig Weight in Grams	Subcutaneous Injection in c.c.	Result	Autopsy	Cultures
Unaltered culture of B. Preisz-Nocard	480	1	After 15 hr., markedly intoxicated. After 24 hr., dying	Hemorrhagic infiltration at site of inoculation. Lungs slightly hyperemic. Slight orchitis on one side	Subcutaneous tissue at site of inoculation, heart blood, and testicle sterile
Sediment washed in salt solution + 0.85% NaCl to volume of original culture	360	1	After 40 hr., slightly ill. On fourth day, local suppurative abscess at site of inoculation. Recovered		
Sediment washed in broth made up to volume with broth	...	1	Same course as that above, except for precrural abscess. Recovered		
Berkefeld filtered supernatant fluid *	440	10	Dead in 15 hr.	Slight hyperemia at site of inoculation. Lungs slightly inflamed. Peritoneal surfaces markedly hemorrhagic with red serum in cavity	Subcutaneous tissue, heart blood, and peritoneum sterile
	360	1	Markedly intoxicated; dead after 22 hr.	Slight edema at site of inoculation	Subcutaneous tissue and heart blood sterile
	360	1 (1:10 dilution in 0.85% NaCl)	No symptoms		

* No preservative added to this fluid before Berkefeld infiltration. Tested for sterility by inoculation of 5 c.c. into blood agar, 4 days' incubation.

The essentials of this experiment were corroborated with Strain III with even more convincing results. In this instance 1 c.c.

of culture, washed free of toxin by 4 successive centrifugations and decantations, failed to intoxicate acutely a guinea-pig, tho it proved fatal in 28 days. A 0.1-c.c. dose of unaltered culture proved fatal in about 48 hours, with marked intoxication and the usual appearance at autopsy. The supernatant fluid was fatally toxic in 24 hoiurs for a guinea-pig injected intraperitoneally with 1 c.c., but 0.1 c.c. failed to kill another one similarly inoculated.

Our experiments leave no doubt as to the formation of a weak soluble toxin by B. Preisz-Nocard. The strongest toxin we have secured was by centrifugation of a culture to which 0.4% tricresol had been added. This certainly renders the supernatant fluid sterile, as we have found by cultural test. Such a toxin, prepared from Strain XV, sufficed in a dose of 0.2 c.c. to intoxicate fatally a 220-gram guinea-pig in less than 48 hours. The toxicity deteriorates rapidly, however, even in the ice-chest, for we found 3 months later that 0.5 c.c. was non-fatal, tho 1 c.c. still proved lethal.

We believe that the toxin and the hemyolsin are not identical, because most of our toxic filtrates were non-hemolytic; moreover, the toxicity was destroyed by 5 minutes' boiling, whereas the hemolytic properties of certain supernatant fluids were not injured by boiling.

ANTITOXIC IMMUNITY

Antitoxin effective experimentally in a dose of 1 c.c. against 3 c.c. of toxin when tested upon guinea-pigs, was prepared by Carré and Bigoteau,[20] but it was incapable of preventing the appearance of abscesses or arresting the toxic affection of lambs known in France as "eaux rousses"; they also found sheep—well immunized against the toxin—not immune to pyogenic infection by the bacillus. Of unique interest from an academic viewpoint is a single case mentioned by Valleé[21] as quickly aborted by the use of diphtheria antitoxin. In view of the ready spontaneous healing of lymphangitic ulcers properly drained, this declaration might pass unheeded were it not supported by the careful experimentation which led Dassonville[19] to the conclusion that "le sérum anti-diphtérique paralyse l'action de la toxine du bacille de Preisz-Nocard; il en retarde considerablement les effets, parfois d'une façon indéfinie." Dassonville found that to render one fatal dose of the toxin of B. Preisz-Nocard inactive, more than 250 times as much diphtheria antitoxin was required as for one fatal dose of diphtheria

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[21] Bull. d la Soc. centr. de Méd. vétérin, 1907, 84, p. 181.

toxin. In other words, it is claimed that while the toxin of B. Preisz-Nocard resembles that of diphtheria, it is not identical with it, and that the neutralization thereof by diphtheria antitoxin is partial only.

We might cite a number of experiments demonstrating the partial neutralization of B. Preisz-Nocard toxin by diphtheria antitoxin, but we would rather withhold positive statements regarding this matter for more complete proof. We did not study the action of serum from non-immunized horses upon B. Preisz-Nocard toxin, but we might recall that Dassonville[19] failed to find any evidence of protection thereby. An aggravating element in such a study might be the rapid attenuation of the comparatively weak toxin of the bacillus of Preisz-Nocard which we have experienced.

BACTERIAL IMMUNITY

Having a number of guinea-pigs which had survived previous experiments, we were interested in making tests of their resistance to further infection. Thus of 4 male guinea-pigs, weighing from 440 to 700 grams, which several weeks before had received 10 c.c., 0.1 c.c., and 0.02 c.c., respectively, of B. Preisz-Nocard III filtrate without percepti-ble results, 3 died within 48 hours when injected subcutaneously with 1 c.c. of a 3-day culture of this strain in glycerin broth. Pure cultures were recovered from the subcutaneous tissues, but heart blood gave no growth upon blood agar. A fourth guinea-pig was also intoxicated but recovered to suffer from a localized subcutaneous abscess, which soon healed completely.

Four other guinea-pigs were injected subcutaneously with 1 c.c. of the same culture at the same time, all having received previously cul-tures or emulsions of bacilli ranging from 0.001 c.c. to 1 c.c., and 3 had apparently recovered from suppurative lesions involving for 2 of them complete emasculation. The third, Guinea-pig 124, a female, had received 1000 units of diphtheria antitoxin with the original infection and had displayed only a local abscess of slight severity. The fourth animal had become emasculated and had still a swollen precrural gland. In none of these animals did the new injection cause acute intoxication, but all developed the local subcutaneous edema characteristic of animals injected with the toxin of B. diphtheriae and of B. Preisz-Nocard. The two heaviest recovered easily within 15 days. The lightest one, Guinea-pig 124, suffered for over 4 weeks from localized and metastatic abscesses, while the heavier one had the existing swelling in the pre-crural gland rapidly exacerbated to the point of suppuration, in addition

to the formation of an abscess at the site of inoculation. It had apparently recovered, however, within one month after the inoculation.

A further immunity test was made upon these guinea-pigs by injecting them intraperitoneally, together with 3 others, of which 2 still suffered from suppurative processes, with 5 c.c. of a 7-day glucose broth culture of B. Preisz-Nocard II. This test, a severe one, intoxicated all of the animals under observation, except Guinea-pig 124, and killed acutely some of those which might, from their previous resistance, have been considered quite immune. Others apparently recovered, but in certain instances only after a severe course of recurrent abscesses, particularly of the testicular remnants or of the lymphatic nodes.

The findings at autopsy in some of these animals are worthy of special mention.

Thus, Guinea-pigs 110, 112, and 117, comprising those acutely and fatally intoxicated, showed in each case a hemorrhagic condition of the lungs; a diffuse, purulent peritonitis, particularly localizing in and invading the spleen; and a marked hyperemia and enlargement of the suprarenal capsules.

Guinea-pigs 103 and 111, apparently on the way to recovery, died unexpectedly during a sharp, frosty spell of weather. The lungs of the former were hemorrhagic and the suprarenals were hyperemic, but the only pus discoverable was in the testicular vestiges and this contained B. Preisz-Nocard in pure culture. Both animals were emaciated, particularly Guinea-pig 111. In the latter there were practically complete destruction of the spleen, as well as the testicles, and deep congestion of the suprarenal capsules, which were enlarged nearly to the size of the kidneys.

These observations show that a variable degree of immunity is conferred by an attack of lymphangitis in guinea-pigs. In the practical immunization of large animals effort should be directed toward antitoxic, as well as bacterial, immunity. The latter seems to us, however, to be more important in ulcerative lymphangitis than in either diphtheria or tetanus.

SUMMARY

We have isolated B. Preisz-Nocard from characteristic abscesses in 11 horses and one calf. The etiology of the lesions from which it was obtained is identical with that of caseous lymphadenitis of sheep, and the disease in horses known as ulcerative lymphangitis should be differentiated by laboratory diagnosis from farcy, epizootic lymphangitis, and sporotrichosis, all of which have a mutual resemblance clinically.

B. Preisz-Nocard is a diphtheroid bacillus, presenting interesting characteristics as follows: (1) the production of orchitis in guinea-pigs, as well as suppurative processes generally throughout the lymphatics; (2) the hemolysis of blood agar plates not containing an excess of fermentable carbohydrate; (3) the elaboration of a soluble toxin, resembling but not identical with that of diphtheria, yet being neutralized partly by diphtheria antitoxin. This apparent partial neutralization suggests the existence of group reactions among soluble bacterial toxins analogous to the group reaction of precipitins and agglutinins.

We again draw attention to the uncertainty of experimental orchitis in guinea-pigs as a certain test for glanders, and emphasize the necessity of microscopic and cultural examination of pus from such lesions for diagnostic purposes.

FURTHER INVESTIGATION INTO THE PRECIPITATION OF THE TYPHOID BACILLUS BY MEANS OF DEFINITE HYDROGEN-ION CONCENTRATION *

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The phenomenon of acid agglutination has been the subject of previous investigations by different workers with widely differing results. By acid agglutination is meant the spontaneous clumping of bacteria and other small particles in suspension by means of a definite concentration of the hydrogen ion.

It has been the contention of certain German workers that as definite concentrations have been proved specific for the precipitation of certain organisms, they might therefore be used as a differential test for recognizing certain bacteria; for example, B. typhosus. Other investigators have denied the validity of such conclusions and discouraged the idea that it could ever be of any practical use.

It has been the purpose of our investigations to determine whether there exists a specific hydrogen-ion concentration for the precipitation of all strains of B. typhosus; and, if so, whether this phenomenon could be applied in precipitating B. typhosus from other germs in suspension, and thus in many cases positively proving the presence of this bacillus in suspected waters.

Before giving the methods employed and the results of our investigations, we shall review the methods and conclusions of previous workers.

In the year 1911, Michaelis[1] investigated the phenomenon, using acetic acid. A series of solutions with gradually increasing amounts of the hydrogen ion was prepared as follows:

Solutions	N/NaOH	N/Acetic	Distilled Water	(H)
1.....................................	5 c.c.	7.5 c.c.	87.5 c.c.	1×10^{-5}*
2.....................................	5 c.c.	10 c.c.	85 c.c.	2×10^{-5}
3.....................................	5 c.c.	15 c.c.	80 c.c.	4×10^{-5}
4.....................................	5 c.c.	25 c.c.	70 c.c.	8×10^{-5}
5.....................................	5 c.c.	45 c.c.	50 c.c.	16×10^{-5}
6.....................................	5 c.c.	85 c.c.	10 c.c.	32×10^{-5}

* 1×10^{-5} is the chemical formula for indicating the hydrogen-ion concentration. Written in full it is 0.00001.

[1] Deutsch. med. Wchnschr., 1911, 37, p. 969.

When a suspension of a 24-hour culture of B. typhosus was added to each of the solutions of this series, it was found that the suspension was always precipitated between Solutions 2 and 5 and that the optimal precipitation took place in Solution 3. Therefore, the optimal precipitation took place at a definite hydrogen-ion concentration which, according to Michaelis,[1] was 4×10^{-5}. B. coli was not precipitated in any of the solutions. B. paratyphosus was precipitated in Solutions 5 and 6. B. enteritidis was found to have no definite optimal precipitation point, but might be precipitated at any concentration, depending upon the strain used.

Beniasch,[2] who worked with very many strains of all the well-known pathogenic bacteria, using in each case acetic, lactic, and levulinic acid, concluded that each germ has a specific hydrogen-ion concentration for its optimal precipitation, that the optimal precipitation for B. typhosus is 3.6×10^{-5} regardless of the acid used, and that this concentration could be used for identifying B. typhosus.

Schidorsky and Reim[3] have likewise claimed that this test is of differential value and they claim to have used it in practical work. Jaffé[4] denies that the test is specific and doubts whether it can be used to advantage. Sears[5] used hydrochloric acid, as well as acetic and lactic acid. He also worked with and without the sodium salt of the acid, with some difference, as a result, in precipitation. He found some strains of B. typhosus that were not precipitated anywhere near the (H) of Michaelis and Beniasch.

In our work we made use of 6 strains of B. typhosus. We also used 4 strains of B. coli, 2 strains of B. paratyphosus, 1 strain of B. paracoli, 1 strain of B. enteritidis, and 1 strain of B. dysenteriae. The strains of B. typhosus had all been in culture for a considerable time, most of them being known to be several years old. Altho the laboratory history of each strain indicated that it was a genuine typhoid bacillus, yet each one was carefully tested before beginning the investigations. They all gave typical growths and reactions upon gelatin, potato, milk, litmus lactose agar, dextrose broth, Dunham's peptone solution, nitrate broth, and Endo's medium. They were also agglutinated in immune sera, tho they were not all equally agglutinable.

The sources of the various strains of B. typhosus used were as follows: Strain 1010, isolated several years ago from a patient in the University of Colorado hospital, Boulder, Colorado, has been kept in culture in the department of bacteriology of the University of Colorado. Strain 1012 was secured from the laboratory of the Chicago board of health 5 years ago. It had been isolated from feces. Strain 1013 was secured from Parke-Davis Company, Detroit, Michigan, 2 years ago. Its previous history is not known. Strain 1014 was secured from the Cutler laboratory, Berkeley, California, about 1 year

[2] Ztschr. f. Immunitätsf., 1912, 12, p. 241.
[3] Deutsch. med. Wchnschr., 1912, 38, p. 1125.
[4] Arch. f. Hyg., 1912, 76, p. 1.
[5] Proc. Soc. Exper. Biol. and Med., 1913, 10, p. 120.

ago, previous history being unknown.   Strain 1016 was recently secured from Dr. Philip Hilkowitz' clinical laboratory, Denver, Colorado, its previous history being unknown.   Strain 1017 was secured from the Colorado state agricultural college, Fort Collins, Colorado, its previous history being unknown.   It is not likely that these strains of B. typhosus are closely related, and therefore they may be regarded as fairly representative of all strains of this organism.   We were unable to determine whether the sources of the various strains used by previous workers were thus widely separated, or whether the strains had been isolated from patients of one epidemic, or from one community.

The work was begun by using tartaric acid.   In all the previous work the sodium salt was used to reduce the hydrogen-ion concentration and also to hasten the precipitation.   (No explanation has been given as to why the salt hastens precipitation.)   So we used a salt of tartaric acid,—sodium-potassium-tartrate.   The acid and tartrate were carefully prepared in N/1, N/10, and N/100 solutions and a system of dilutions worked out in test tubes giving the alleged optimal hydrogen-ion concentration, with higher and lower concentrations on either side.   Several such series of test tubes were set up and tested with B. typhosus.   Tho there were some positive results, in the main they were irregular and not at all reliable.

Acetic acid was then substituted for tartaric acid for the following reasons : First, the hydrogen-ion concentration constant of tartaric acid is much higher than that of the acetic acid, and therefore more difficult to control; second, within 24 hours after setting up the system a fungus growth appears in tartaric acid which interferes with normal slow precipitation.   The fungus growth brings down all germs by acting as a filter.   This fungus growth can be prevented only by aseptic care.

In working with acetic acid and sodium acetate a system was set up identical with that used by Beniasch.[2]   The solutions were prepared to give varying hydrogen-ion concentrations in a series.   Table 1 indicates the amounts added to give the (H) in each tube :

The concentration of the hydrogen ion was computed from the formula (H) $=$ K $\times$ (acid), in which "K" equals the dissociation constant.   The constant of acetic acid at room temperature is 1.8×10^{-5} or 0.000018.   By solving for each solution in the series the hydrogen-ion concentration is determined for each tube.   These are given in the table.

TABLE 1

Series of Tubes — Varying (H)

Solutions	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9
N/10 c.c. sodium acetate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Acetic acid c.c. N/10 Tubes 1-5, N/1 Tubes 6-9	0.06	0.12	0.25	0.5	1	0.2	0.4	0.8	1.6
Distilled Water	1.54	1.48	1.36	1.1	0.6	1.4	1.2	0.8	0
24-hr. culture suspension	1	1	1	1	1	1	1	1	1
Total fluid	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
K- 1.8×10^{-5}	.....	.....	...	.....	.....	.....	......	.....	.....
Hydrogen-ion concentration—(H) ...	2.3×10^{-6}	4.5×10^{-6}	9×10^{-6}	1.8×10^{-5}	3.6×10^{-5}	7.2×10^{-5}	1.45×10^{-4}	2.9×10^{-4}	5.8×10^{-4}
B. coli 1002...........	.....	.....	...	.....	.....	.....	......	.....	.....
B. coli 1003...........	.....	.....	...	.....	.....	.....	......	.....	.....
B. coli 1004...........	.....	.....	...	.....	.....	.....	......	.....	.....
B. coli 1005...........	.....	.....	...	.....	.....	.....	......	.....	.....
B. paracoli 1008.....	.....	.....	...	.....	.....	.....	......	.....	.....
B. paratyphosus 1021	.....	.....	...	.....	.....	.....	......	.....	+
B. paratyphosus 1023	.....	.....	...	.....	.....	.....	......	.....	.....
B. dysenteriae 1024..	.....	.....	...	.....	.....	.....	......	.....	.....
B. enteritidis 1026...	.....	.....	...	.....	.....	.....	......	.....	.....
B. typhosus 1010....	.....	.....	...	.....	.....	.....	......	.....	.....
B. typhosus 1012....	.....	.....	...	.....	.....	.....	......	++	++
B. typhosus 1013....	.....	.....	...	++++	++++	.....	......	.....	.....
B. typhosus 1014....	.....	.....	...	+	++++	+++	+	.....	.....
B. typhosus 1016....	.....	.....	...	.....	++	++	+++	.....	.....
B. typhosus 1017....	.....	.....	...	+++	+++	+	+++	.....	.....

The agar used was prepared from Fairchild's culture peptone 1.5% acid. Before inoculating the tube, the water of condensation was poured off to avoid the salt that was in the media. The tubes were inoculated and incubated at 37.5 C. for from 20 to 30 hours, tho as a rule for just 24 hours. A few were incubated for 48 hours.

When the solutions were prepared, the growth from each agar slant was washed off with distilled water and filtered through filter paper to make sure that there were no small particles of agar in the suspensions. About 15 c.c. of the suspension were made from each tube. The suspension was then added to each test tube as indicated in Table 1. Then the series of tubes was placed in the incubator for 1 hour at 37.5 C., after which they were removed and the precipitate noted.

Each of the four strains of B. coli was thus treated, but no precipi tate occurred either immediately or after several days' standing at room temperature. This was repeated 4 times with the same result each time.

The six strains of B. typhosus were also prepared and set up in the same manner, but with quite different results. Strain 1010 failed to be precipitated by any of the concentrations of the hydrogen ion used. This was repeated 3 times, but failed to give a precipitate at any time, either immediately or after several days' standing. Strain 1012 gave a precipitate in Tubes 8 and 9 after several hours' standing, but never immediately after removing from incubator. Twice it gave no

macroscopic precipitation, but microscopically it showed agglutination. This strain, therefore, was slowly and poorly agglutinated and only at relatively high concentrations. Strain 1013 gave a very definite and rapid precipitation in Tubes 4 and 5. It was marked to the naked eye immediately upon removal from incubator. At first, it was in suspension in macroscopic masses, but soon fell to the bottom of the tube. This strain gave the same result upon 3 subsequent trials. Strain 1014 was likewise rapidly and definitely precipitated, but at a slightly higher concentration. Three times it was precipitatetd in Tubes 5 and 6, and once in 4 and 5. Strain 1016 was precipitated slowly, but definitely, within 24 hours. Once it was precipitated in Tube 7 only, and twice in Tubes 5 and 6 as well as 7. Strain 1017 was always rapidly precipitated in 4 and 5. Later it was precipitated in higher concentrations, beginning in 7 before 6,—a very interesting phenomenon which held good each time tried.

B. dysenteriae was tried twice, but was not precipitated anywhere in the system either early or late. The two strains of B. paratyphosus were tried 3 times, but in only one case was there a precipitation and that was in Tube 9 of Strain 1021 after 24 hours' standing. B. paracoli was never precipitated.

With this test we found that one strain of typhoid bacillus acted just like all the strains of B. coli, one was very similar to one strain of B. paratyphosus, while the other four were precipitated at fairly constant hydrogen-ion concentrations. One strain was not precipitated within 2.3×10^{-6} and 5.8×10^{-4} (H). Another strain was precipitated only at 2.9×10^{-4} and 5.8×10^{-4} (H). The four remaining strains were precipitated between 1.8×10^{-5} and 1.45×10^{-4}. These four strains could all be precipitated with 3.6×10^{-4}, if enough time were given.

Each strain was fairly constant in concentration necessary for precipitation. The slight variations can be accounted for in that it is quite impossible to obtain each solution with exactly the same hydrogen-ion concentration every time set up.

It was also observed that those strains which were most rapidly precipitated correspond with the strains which have the laboratory history of being the most rapidly and strongly agglutinated by immune typhoid sera. Strain 1010 is almost useless for agglutination tests with typhoid bacilli. It is likewise unprecipitable in any of the concentrations of the hydrogen ion prepared in the system.

These results agree with those of Sears,[5] but do not agree entirely with the findings of Michaelis[1] and Beniasch.[2] The reason for this disagreement must indicate that these earlier workers employed strains which were closely related, while we, like Sears, worked with strains very distantly related.

The foregoing facts render the application of this phenomenon to the precipitation of B. typhosus from suspected water rather discouraging. However, a few tests were made. To 500 c.c. of distilled water we added the acid and acetate in such proportions as to give 3.6×10^{-5}. Then we added the entire growth from a 24-hour agar slant of B. typhosus 1014 and incubated the whole for 1 hour, as we had done with test-tube preparations. The typhoid bacilli were precipitated, but only after several days, while in the test tube they were precipitated immediately. This delay was probably due to the fact that the suspension was far more dilute than in test tube. In water contaminated with B. typhosus, the bacilli are probably still more diluted than in this test preparation. Besides, these "wild" strains are said to be less agglutinable than those kept for a long while in culture. Moreover, as has been shown by our previous experiments, there is a different concentration for different strains of B. typhosus. Therefore, further work along this line was not pursued.

In making microscopic study of the suspensions of the strains that were not precipitated rapidly and at the supposed optimal precipitation point, we often observed that the bacilli were clumping very like bacilli in an agglutination test with typhoid bacilli, tho they had not yet agglutinated in large enough masses to be precipitated out of the solution. This fact would indicate that pseudo-clumping in an agglutination test with typhoid bacilli might easily be due to traces of acid washed into the hanging drop from the apparatus used in its preparation.

CONCLUSIONS

There is no definite hydrogen-ion concentration which will precipitate all strains of B. typhosus.

Tho there is a fairly wide range in hydrogen-ion concentration for the precipitation of the various strains of B. typhosus, most strains can be precipitated in the presence of 3.6×10^4 hydrogen-ion concentration, derived from the dissociation of acetic acid in the presence of sodium acetate.

Those strains that are easily and rapidly agglutinated by means of immune sera are likewise easily and rapidly precipitated by the hydrogen ion, probably of the concentration 3.6×10^{-4}.

The hydrogen-ion test may sometimes aid in the determination of B. typhosus. However, it can never be a certain test.

It cannot be used as a practical method of precipitating B. typhosus from contamiated waters.

It indicates a possible source of error in performing an agglutination test with typhoid bacilli.

A LEPTOTHRIX ASSOCIATED WITH CHRONIC HEM-ORRHAGIC NEPHRITIS *

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In addition to cases of hematuria associated with known causes, such as tuberculosis, stone, tumor, etc., there occasionally occur cases of hematuria of obscure etiology, known as "essential hematuria," "renal epistaxis," or "renal hemophilia." On account of its clinical resemblance to such conditions, the case here reported is of interest.

A German girl, 15 years old, was admitted to the Durand Hospital with scarlatina. She had been well, except for measles and whooping cough, until her twelfth year; then she had been confined to bed for 2 weeks with her first attack of hematuria. She had been skating and had caught cold. The cold had been followed by sore throat, fever, pain in both lumbar regions, and bloody urine. Thereafter at intervals of 3 to 30 days, usually only a few days, she had had attacks of general malaise and aching in the lumbar region, followed by large amounts of blood in the urine.

Two years after the onset of hematuria she had had diphtheria, and during the 6 weeks of quarantine the bleeding had been severe. On being released from quarantine she had sought medical advice. A thorough examination, including cystoscopy and ureteral catheterization, had revealed nothing pathologic except blood coming from both kidneys. After a fruitless search for tubercle bacilli, a diagnosis of chronic nephritis had been made. The onset of scarlet fever had been preceded by an attack of hematuria.

On admission there was tenderness in both lumbar regions. The systolic blood pressure was 102. There was a fading scarlet rash.

The urine, which was dark reddish-brown and turbid, gave a heavy precipitate of albumin and showed in a drop of the uncentrifugated specimen many erythrocytes, blood casts, and an occasional finely granular cast. Polymorphonuclear leukocytes were comparatively few in number.

Repeated examinations for tubercle bacilli (simple smear and antiformin preparations) gave negative results. Cultures of 2 c.c. of uncentrifugated urine in dextrose agar shakes made at the time of admission and again 9 days later, yielded the organisms to be described in about the same numbers.

Leptothrix.—From 2 c.c. of urine there grew innumerable small colonies of gram-positive organisms, varying from short bacillary forms to long, tangled threads, the thread form predominating. The organism showed neither true nor false branching and did not have a sheath. It was anaerobic and grew slowly. On blood ascites agar, the colonies, as they first appeared, were transparent points, which by the end of a week had coalesced, forming a thin, brownish, opaque film. Growth on human blood agar was more luxuriant than on goat's blood and human ascites fluid agar. The blood was not affected by the growth of the organisms. On ordinary media the growth was too slight to give satisfactory cultural reactions.

Yeast.—From each 2 c.c. of urine there developed 3 to 5 large, fuzzy, white colonies of an aerobic yeast, which grew only on dextrose-containing media. Inoculated intravenously and subcutaneously into a rabbit, the yeast caused no lesions.

The leptothrix growth from 4 agar slants was injected into the marginal vein of a rabbit's ear. The animal died in convulsions almost at once. A second rabbit, inoculated with the growth of leptothrix from one agar slant, like the first rabbit died immediately. Attempts to produce disease in rabbits with smaller amounts of the leptothrix, injected intravenously and subcutaneously, failed. Animal experiments were then discontinued, as the organism had apparently lost its toxicity, even in dosage exceeding that in which it was fatal when first isolated.

The patient received the following doses of a vaccine from the leptothrix: on April 12, 1 million organisms; on April 14, 2 million organisms; and on April 24, 4 million organisms.

After the third dose, the patient reported that the urine, which had been macroscopically free from blood, became very bloody for 3 days. Tho she had previously had attacks characterized by general malaise and large amounts of blood in the urine at intervals of 3 to 30 days, another one did not occur during the next 16 weeks. The dose of vaccine was reduced; an injection of 2 million organisms was made on May 8; and again on May 15.

On microscopic examination red cells were still found in the urine after the fourth and fifth injections and the dose of vaccine was further reduced; injections of 500 thousand organisms each were made on May 22, May 29, June 5, June 19, and June 26, respectively.

After the ninth injection no erythrocytes were found in the urine;
it still showed an occasional hyaline or granular cast.   The patient
meanwhile had gone back to work and had resumed active outdoor
exercise.   The urine remained free from red cells during the following
9 weeks that she was under observation.   Injections of one-half-million
doses at intervals of 1 to 2 weeks were continued.   On July 31 the
urine contained a few hyaline casts.   On August 21 several hyaline
casts and an occasional granular cast were found.
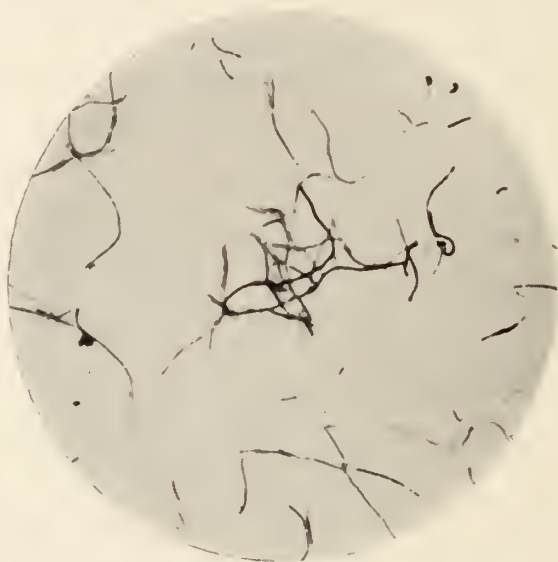
Fig. 1.   Photomicrograph of Leptothrix in pure culture.

The exact classification of organisms of the group Trichomycetes,
which have been observed in a variety of diseases, is made somewhat
difficult by the confusion in nomenclature.   The classification followed
by us is that given by Lehmann and Neumann.[1]

Arustamow[2] isolated a leptothrix from the urine of a tabetic, but it is not
clear from the abstract of the original article, which is Russian, that the organ-
ism was associated with any disease of the urinary tract.   Flexner[3] isolated a
pathogenic leptothrix from a puerperal infection of a rabbit.   Leptothrix infec-
tions of the throat have been described (pharyngomycosis leptothrica, Chiari;

1. Bakteriol. Diagnostik, 1907, 2, p. 588.
2. Centralbl. f. Bakteriol., 1889, 6, p. 349.
3. Jour. Exper. Med., 1905, 1, p. 211.

mycosis tonsillaris benigna, B. Frankel), and it is interesting to note that in the case now reported the first attack of hematuria was preceded by a sore throat.

Our case may be summed up as a chronic nephritis, characterized by frequently recurring attacks of hematuria extending over a period of 3 years, and by Leptothrix in large numbers in the urine. The presence of the leptothrix in the urine is not proof that it caused the nephritis and hematuria with which it was associated. But the large numbers in which it was found, its toxicity for animals, and the marked improvement of the patient during vaccine treatment suggest a causal relation.

OBSERVATIONS UPON THE ENDAMEBAE OF THE MOUTH *

I. ENDAMOEBA GINGIVALIS (BUCCALIS)

PLATE 6

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As a result of the statements of several recent writers and investigators that the endamebae so frequently encountered in the human mouth are pathogenic organisms and the cause of pyorrhea alveolaris and of many of the complications of so-called "oral-sepsis," interest has been reawakened in the study of these parasites, and several interesting and valuable contributions have appeared dealing with Endamoeba gingivalis (buccalis), a species commonly observed in the human mouth and held by some writers to be the cause of pyorrhea.

I have accepted the spelling Endamoeba instead of Entamoeba because of the fact that Joseph Leidy established the genus Endamoeba in 1879, to include parasitic amebae, the type species of the genus being Endamoeba blattae, of the cockroach. The genus Entamoeba was established in 1897, by Casagrandi and Barbagallo, who were evidently ignorant of Leidy's genus Endamoeba for parasitic amebae. By virtue of the law of priority the spelling of Leidy is to be preferred to that of Casagrandi and Barbagallo, and for this reason I adopt the name Endamoeba in place of Entamoeba.

During the past 9 months, through the kindness of Dental Surgeon Minot E. Scott, U. S. Army, stationed at this post, I have had the opportunity of studying the endamebae occurring in the mouth in patients suffering from pyorrhea alveolaris and other inflammatory conditions of the teeth and gums, and have also studied the endamebae occurring in normal mouths, and in this contribution I shall describe the morphology and life cycle, so far as I have been able to determine it, of Endamoeba gingivalis, Gros, which I believe to be identical with

Endamoeba buccalis, Prowazek. In a future contribution I hope to be able to describe the complete life cycle of what I believe to be a new species of endameba, occurring in this region, which I have provisionally named Endamoeba confusa. This species is characterized by its minute size, the fully developed vegetative organisms seldom measuring over 10 microns in diameter; the slight distinction between ectoplasm and endoplasm; a nucleus of the histolytica type but very minute; very sluggish motility under the most favorable conditions; and the absence of ingested bodies, which are so very frequently observed within Endamoeba gingivalis. This specific name "confusa" is given because of the liability of confusing this species with the smaller examples of Endamoeba gingivalis.

In all probability, further research will demonstrate that still other species of amebae occur in the human mouth, and it will be strange indeed if both Endamoeba histolytica and Endamoeba coli, the common intestinal endamebae of man, are not sometimes encountered in this locality, for it is through the mouth that infection of the intestine with these parasites occurs. It is also probable that some of the species of purely vegetative amebae will be found in the mouth, especially those belonging to the genus Vahlkampfia, for these have been found in the intestine of man, reaching that locality through the mouth. The probability of the occurrence in the mouth of these various species of amebae should not be lost sight of in the study of this subject and for this reason one should be very cautious in drawing conclusions regarding the species of amebae encountered in this region and their relation to diseases of the teeth, gums, and adjacent structures.

I. Endamoeba gingivalis Gros 1849. em. v. Prowazek 1904

This, in all probability the most common species of endameba occurring in the mouth whether in health or disease, has recently attracted much attention because of its supposed relation to the etiology of pyorrhea alveolaris, and for this reason a detailed description of its morphology and life cycle is of interest to both physicians and dentists, especially as many of the descriptions in the literature are very brief and of little value to one who desires to differentiate this species from other species of endamebae.

History

Gros,[1] in 1849, described an ameba occurring frequently in the soft tartar of human teeth, characterized by blunt pseudopodia; a clear distinction between the ectoplasm and the endoplasm; the presence of ingested bodies, or globules,

[1] Bull. Soc. imp. de nat. de Moscow, 1849, 22, p. 549.

within the cytoplasm; and ameboid motility. The size of the organism, according to Gros, varied from 25 to 30 microns in diameter. He named the parasite Amoebea gengivalis.

In 1862, Steinberg[2] described an ameba which he found in the soft tartar and in the refuse material around the teeth, which, so far as his description indicates, was identical with the organism described by Gros, altho he evidently considered it a new species and named it Amiba buccalis. The same organism was again described as a new species by Grassi,[3] in 1879, who found it in material from the lesions of gingivitis. He named it Amoeba dentalis.

In 1904, v. Prowazek[4] gave a detailed description of an endameba occurring in the mouth, which he considered a new species, and which he named Entamoeba buccalis, evidently in ignorance of the prior use of this specific named by Steinberg in his description of the ameba found by him in the mouth, in 1862. Von Prowazek described the endameba observed by him as varying in size from 6 to 32 microns in diameter; actively motile; having a clear, distinct ectoplasm and an endoplasm containing many food vacuoles and much ingested material. The nucleus measured from 1.5 to 4.5 microns in diameter, was poor in chromatin, and had a well-defined, thick nuclear membrane and a deeply stained, very minute karyosome. Reproduction occurred by simple division, mitosis occurring first in the karyosome; but Prowazek also suggested that reproduction might occur by budding or gemmation, as he found in the endoplasm what he believed to be chromidia of nuclear origin. He was unable to find any reproduction cysts. He states that this parasite may be differentiated from Endamoeba coli by the clear hyaline ectoplasm and the differences in reproduction, and from Endamoeba histolytica by the very definite nuclear membrane and the greater rigidity of the nucleus.

Altho Prowazek gave a better description of this endameba than his predecessors, it is very evident that his Entamoeba buccalis is identical with the organism described previously by Gros, Steinberg, and Grassi, and it therefore follows that the specific name "buccalis" must be replaced by the name "gingivalis," first given the parasite by Gros.

In 1907, Lewald[5] stated that he had been able to demonstrate this endameba in the mouths of 75 of 100 individuals examined, and that they occurred in this region no matter how much care was given to the teeth.

But little attention was given Endamoeba gingivalis until the appearance of a paper by Barrett[6] upon the protozoa of the mouth in relation to pyorrhea alveolaris. In conjunction with Allen J. Smith he examined 46 patients suffering from suppurative conditions of the gums and the pericemental tissues and found Endamoeba gingivalis in all of them. He concludes the paper by stating his belief in the etiologic relation of this parasite to the lesions of pyorrhea and notes their disappearance from the lesions after the administration of emetin, a well-known amebacide.

Barrett's paper was followed by an important one from Chiavaro,[7] who studied this endameba under the direction of Grassi, in the latter's laboratory. He states that in all probability the organisms studied by Gros, Steinberg, Grassi, v. Prowazek, and himself are identical. Chiavaro is the first to describe

2 Souremenaya meditsina., Kiev, 1862.

3 Gazz. med. Ital.-Lomb., 1879, 39, p. 446.

4 Arbeit. a. d. k. Gsndhts., 1904, 21, p. 42.

5 Proc. New York Path. Soc., 1907. Cited, New York Med. Jour., 1915, 102, p. 281.

6 Dent. Cosmos, 1914, 56, p. 948.

7 Dent. Rev., 1914, 27, p. 1122.

encysted forms for Endamoeba gingivalis. He observed encystment in one case but gives no detailed description of the cysts, altho he pictures a cyst containing 2 nuclei. His drawings of the cystic forms are far from convincing, but it may be that he really observed the resistance cysts to be hereafter described.

In a later paper, Smith, Middleton, and Barrett[8] describe the occurrence of Endamoeba gingivalis in the tonsils in cases of chronic tonsillitis; and Bass and Johns,[9] as the result of their observations of this organism, concluded that it is the cause of pyorrhea, but added nothing to our knowledge of its morphology or life-cycle. In fact, the method of staining employed by them, as described in their paper, is valueless from a morphologic standpoint; with it one would be unable to differentiate this species from other species that occur in the mouth. From the description of the morphology of the parasite as given by them one is unable to be sure that they did not base their conclusions upon the observation of more than one species, while their unqualified statement that pyorrhea is due to Endamoeba gingivalis (buccalis) is entirely unjustified by any evidence that they were able to present in their contribution.

Several papers have very recently appeared in the dental journals dealing with the relation of this parasite to pyorrhea and other inflammatory conditions of the teeth and gums, the most important being those of Henrici and Hartzell,[10] Sarrazin,[11] and Price and LaRue,[12] and their conclusions will be discussed in that section of this contribution dealing with the relation of Endamoeba gingivalis to disease.

The most recent paper treating of Endamoeba gingivalis is that of Smith and Barrett,[13] in which they review the papers of the various investigators who have studied this parasite, and give a detailed description of its morphology and methods of reproduction. They agree that Endamoeba buccalis of v. Prowazek, is identical with the organism studied previously by Gros, Steinberg, and Grassi, and rightly state that by law of priority it should be called Endamoeba gingivalis instead of the commonly used name, Entameba buccalis. They state that they have observed resistance cysts, but no reproductive cysts. They believe that Endamoeba gingivalis is with great difficulty differentiated from Endamoeba histolytica and bring up the question of the possible identity of the two organisms.

GEOGRAPHICAL DISTRIBUTION

Endamoeba gingivalis apparently has a world-wide distribution, instances of infection having been reported wherever the parasite has been searched for. I, personally, have observed infections in individuals from many of the States of the Union and from the Philippines. It is more than probable that a careful examination of the natives of all countries would result in the demonstration of the presence of this parasite in the vast majority, and that such examinations would throw considerable light upon the question of the relation of this endameba to diseases of the teeth and gums.

[8] Jour. Am. Med. Assn., 1914, 63, p. 1746.
[9] Ibid., 1915, 64, p. 554.
[10] Jour. Nat. Dent. Assn., 1915, 2, p. 123.
[11] Items of Interest, 1915, 37, p. 404.
[12] Jour. Nat. Dent. Assn., 1915, 2, p. 143.
[13] Jour. Parasitol., 1915, 1, p. 159.

In considering the morphology of Endamoeba gingivalis it will be
necessary to describe its appearance in the living condition and in
stained preparations, and in order to differentiate the organism from
other endamebae it is necessary to employ some method of wet fixation
and staining, for in the living condition this species is differentiated only
with extreme difficulty from certain stages in the life cycle of Enda-
moeba histolytica.

The life-history of Endamoeba gingivalis has not been completely
worked out, but I have been able to determine that it passes through
a vegetative stage of existence, during which it multiplies by simple
division; a pre-cystic stage, in which I have not been able to detect
any evidence of multiplication; and a cystic stage, in which, so far as I
have been able to observe, no development occurs, the cysts at no time
containing more than one nucleus. While it is too early to state
definitely that reproduction cysts do not occur in this species of enda-
meba, the only cysts that I have observed have been resistance cysts,
evidently intended to protect the parasite under unfavorable conditions
for development in the ordinary vegetative stage. This fact, of course,
definitely differentiates this species from Endamoeba coli, in which
cysts are produced containing from 8 to 16 nuclei, and from Endamoeba
histolytica, which produces 4-nucleated cysts, and connects the species
with the free-living amebae, in which, under normal conditions, only
resistance cysts are formed.

Morphology of Endamoeba Gingivalis in the Living Condition

The Vegetative Stage.—The vegetative forms of Endamoeba gingivalis in
the living condition, vary in size from 7 to 35 microns in diameter, the aver-
age size being from 12 to 20 microns in diameter. Most authorities state that
the size varies from 6 to 30 microns in diameter, and Smith and Barrett, in
their latest paper, give the diameter as from 30 to 35 microns. I believe that
most of the organisms in this stage of development that have been described
as measuring less than 10 microns in diameter were not Endamoeba gingivalis
but Endamoeba confusa, the new species I have already referred to, which seldom
measures more than 10 microns in diameter, while the smaller individuals often
measure from 5 to 6 microns in diameter. As regards the very large forms
of Endamoeba gingivalis described, measuring from 40 to 60 microns in diame-
ter, I am inclined to believe, with Smith and Barrett, that such forms are simply
anomalous individuals; but the possibility of the occurrence of both Endamoeba
coli and Endamoeba histolytica in the mouth should be remembered in differen-
tiating these large forms of a species which normally is considerably smaller
than either of the parasites named.

When resting, Endamoeba gingivalis is generally circular in shape, altho it may be irregular, but when in motion it is always irregular in outline. Oval forms are frequently observed, especially in stained preparations.

Motility, under favorable conditions, is marked in this species, but I am unable to agree with Smith and Barrett that motility is as marked as in Endamoeba histolytica. While, as a rule, motility is more pronounced in this species than in Endamoeba coli, I have never observed the very active motility commonly noted in Endamoeba histolytica. As a matter of fact, Endamoeba gingivalis is a rather sluggish species, the vast majority of the organisms simply sending out pseudopodia with little or no progressive motion. Examples, however, are observed which have a rather rapid progressive motion, in a definite direction; in such instances the pseudopodia are finger-shaped and longer than those usually observed.

The cytoplasm of Endamoeba gingivalis is divided into a clear outer portion, the ectoplasm, and a granular inner portion, the endoplasm. These two portions are plainly visible only when motility is present, the ectoplasm forming the pseudopodia. At such times there is a clear distinction between ectoplasm and endoplasm, but in this species the ectoplasm is much less refractive than it is in Endamoeba histolytica, approaching in appearance the ectoplasm of Endamoeba coli.

The pseudopodia, which are always formed by the ectoplasm, vary much in shape. Sometimes, when motility is most pronounced, the pseudopodia are long and slender, or finger-like, while at other times they are very short and blunt. Often endamebae are observed in which several pseudopodia are extruded at the same time, and when this occurs, the pseudopodia are always small and lobose in character and there is little or no progressive motion. In the vast majority of instances the pseudopodia are short and blunt, finger-shaped pseudopodia being observed only in organisms that are actively motile.

When the parasite is moving, the endoplasm flows into the ectoplasmic pseudopodia, the distinct division between these two portions, which exists as the pseudopodia are extruded, disappearing gradually, until, when motion ceases, there is no distinction between ectoplasm and endoplasm. Under high powers of the microscope, the ectoplasm is finely granular in appearance.

The endoplasm contains the nucleus, numerous food vacuoles, and ingested bodies of various nature. No contractile vacuole has been demonstrated by any observer.

In the living condition, in the vast majority of instances, the nucleus is invisible, this species resembling, in this respect, the histolytica stage of development of Endamoeba histolytica. Not infrequently an obscure body may be observed within the endoplasm which might be interpreted as the nucleus, but it is only very rarely that a definite nucleus can be demonstrated in the living organism. When the nucleus can be seen, it consists of a refractive ring of dots forming the nuclear membrane, within which may sometimes be seen one or more refractive granules.

The endoplasm contains numerous food vacuoles, which vary greatly in size in the same organism and generally contain granular material representing the remains of ingested bodies that have been partially digested. The smaller endamebae are less vacuolated than are the large organisms and the vacuoles are generally much smaller, while it is not rare to observe small endamebae of this species which are apparently free from vacuoles.

Endamoeba gingivalis is evidently very actively phagocytic, as shown by the fact that the vast majority of individuals contain numerous ingested bodies,

the most common of which are the nuclei of leukocytes, bacteria, and round or oval bodies, probably of protozoan nature. While this species has been described as actively phagocytic for red blood corpuscles, it has not been my experience that these cells are often present in the endoplasm, even tho the material containing the endamebae also contains blood. Altho there is no doubt that this species can phagocytose red blood corpuscles, it cannot be said that this occurs with anywhere near the frequency that it does in Endamoeba histolytica.

Small, round, and oval bodies are very frequently observed within the endoplasm which are obviously neither erythrocytes nor the nuclei of leukocytes, and some of the endamebae are literally packed with them. The exact nature of these bodies I have been unable to determine, but I believe that they represent some species of protozoan organism, and that they form a considerable part of the food supply of the parasite. Most authorities have apparently interpreted these bodies as the nuclei of leukocytes, but I see no reason for such interpretation, for there is certainly little resemblance between them and the nuclei of leukocytes when they are studied in stained preparations.

In the living endamebae I have never observed changes in the organism which I could be sure were reproductive in character. Rarely two organisms are observed side by side and this phenomenon might be interpreted as the result of division, but such an interpretation would only be a guess. However, I have observed a process which I regard as conjugation, in which two of the endamebae became united and there was a distinct interchange of cytoplasmic material, after which the organisms separated. This interchange of cytoplasmic substance was accompanied by very definite and marked currents within both endamebae, and ingested bodies within the organisms could be observed passing from one into the other.

I have also observed the process described by v. Prowazek and Lowenthal as reproduction by budding or gemmation, in which small particles of cytoplasm are apparently budded off from the periphery of the endameba. The process is exactly similar to that occurring in Endamoeba histolytica which for so long was interpreted as a method of reproduction, but which is now known to be degenerative in character. I am convinced that the same interpretation holds in the case of Endamoeba gingivalis—that organisms in which it is observed are not undergoing reproduction, the changes being due to degeneration of the parasites.

The Pre-Cystic Stage.—In this stage of development, in the living condition, the morphology of this species varies considerably from that observed in the vegetative stage. While I have chosen to call this a definite stage of development, it should be understood that, as yet, I have observed no evidences of multiplication in this stage, and that it is more than probable that the endamebae classified as belonging to this stage are simply organisms which are preparing to encyst.

In the living condition, the pre-cystic forms of Endamoeba gingivalis are markedly reduced in size, the average diameter seldom exceeding 10 to 12 microns. The cytoplasm is generally clear and free from vacuoles and ingested bodies, while a fairly definite nucleus is often observed, the nuclear membrane being represented by a circle of very refractive granules and the karyosome by a single refractive mass at or near the center of the nucleus. There is no differentiation of the cytoplasm into ectoplasm and endoplasm, and motility is practically absent. Rarely very small, blunt pseudopodia may be sluggishly extruded from the periphery of the organism, but when this occurs it will be noted that they are of the same appearance as the endoplasm. In the vast majority of instances

the pre-cystic forms are absolutely immotile, perfectly circular in shape, and composed of a clear, finely granular cytoplasm in which a nucleus, of the character just described, may or may not be visible.

The Cystic Stage.—The formation of resistance cysts occurs in this species, but I have never observed any evidence of reproduction within these cysts, so that I believe that cystic formation in Endamoeba gingivalis is a purely protective process and not a reproductive one, as in Endamoeba coli and Endamoeba histolytica.

In the living condition the cysts of Endamoeba gingivalis measure from 8 to 10 microns in diameter, as a rule, altho larger cysts are sometimes observed. They are perfectly circular in shape, and have a thin cyst wall, which may present a distinct double outline. The cytoplasm is clear and finely granular in appearance and there is no distinction into ectoplasm and endoplasm. A nucleus may or may not be distinguished within the cyst in the living condition, but in stained preparations a single nucleus is always present, except in those cysts that are degenerative in character.

Both pre-cystic and cystic forms of Endamoeba gingivalis occur very rarely, in my experience, and this fact may explain why cystic formation has only been noticed by one or two observers prior to this publication.

Morphology of Endamoeba Gingivalis in Stained Preparations

While the study of living examples of Endamoeba gingivalis is of interest and value, the differentiation of this species from other endamebae, especially from Endamoeba coli and Endamoeba histolytica, is practically impossible, in many instances, unless stained preparations be studied. While one may make a diagnosis of the presence of endamebae by the examination of unstained material from the teeth and gums, it is practically impossible to be sure of the exact species present unless stained preparations are also studied, and to be of value in this direction these must be made by some method of wet fixation and hematoxylin staining. The method that I have found most useful and, at the same time, easiest of application, is fixation in Schaudinn's sublimate-alcohol solution and staining with the Rosenbusch-Hartmann stain for amebae. This method of staining was first brought to my attention by Major F. F. Russell, of the Medical Corps of the Army. It is much more convenient and simple than the other hematoxylin methods that I have employed heretofore. In fact, it is so easy of application and the results are so uniform that it may be employed by anyone at all familiar with laboratory technic, and it entirely obviates the necessity of resorting to such staining methods as Giemsa's or Wright's, preceded by dry fixation, which, because they have been considered less difficult of application, have been largely used in the past in the study of amebae. It is just as easy to employ the method of wet fixation and staining here recommended for amebae as the method advocated by Bass and Johns for the diagnosis of endamebae in the mouth, while this method has the very great advantage that, using it, one is able to differentiate the species of endamebae encountered, whereas with the method described by these writers it is impossible to do so.

The method is as follows:

1. Rapidly spread the material to be examined upon a glass microscopic slide or cover-glass, and before it has had time to dry, immerse the slide in Schaudinn's sublimate-alcohol fixing fluid, prepared as follows: Perchlorid of mercury is dissolved in boiling normal salt solution until a saturated solution

is obtained. Two parts of this solution are added to one part of absolute alcohol, the mixture is warmed, and the preparations are immersed in it for from 2 to 5 minutes.

2. Wash for a few minutes in 70% alcohol; 70% alcohol plus enough iodin to give it a port wine color; 70% alcohol; and distilled water.

3. Place preparations in a 3.5% solution of iron-alum in distilled water. In this they may remain over night, if necessary, but ½ hour is sufficient.

4. Wash thoroughly in distilled water.

5. Stain the preparations with the following lithium carbonate hematoxylin stain:

(a) A 1% solution of hematoxylin in 95% alcohol. This solution should not be used until 10 days after mixing.

(b) A saturated solution of lithium carbonate in distilled water. The two solutions are mixed for staining in the following proportion; of a, 10 c.c., and of b, 5 to 6 drops. The smears are stained with this mixture for from 5 to 20 minutes.

6. Wash thoroughly in distilled water.

7. Differentiate with a weak iron-alum solution. The solution given in Step 3 diluted with 3 parts of distilled water is recommended.

8. Wash thoroughly in distilled water; 95% alcohol; absolute alcohol; clear with xylol, and mount in neutral balsam.

At no time during the fixing and staining process should the preparations be allowed to dry or they will be valueless.

With the method described, Endamoeba gingivalis presents certain characteristics that distinguish it from other species of endamebae that I have studied. As the morphology of the parasite varies at different stages of development, it will be necessary to consider its appearance in the vegetative, pre-cystic, and cystic stages of development separately.

The Vegetative Stage of Development.—Stained specimens at this stage measure from 8 to 25 microns in diameter, altho larger forms may sometimes be encountered. The shape of the organism is usually round or oval, but may be irregular when fixation has occurred during the protrusion of a pseudopodium. There is no distinction between the ectoplasm and the endoplasm in the round or oval organisms, altho in the irregular forms the outer portion, or ectoplasm, forming the pseudopodium, stains a lighter gray than the endoplasm and appears very finely granular. With the staining method described the cytoplasm stains a brownish gray when the specimens are properly differentiated.

The nucleus, in stained preparations, is, in my opinion, wholly characteristic, and easily distinguished from the nucleus of either Endamoeba coli or Endamoeba histolytica, in both size and structure. It is generally situated very near the center of the parasite, but is often also at some distance from the center. It consists of a definite nuclear membrane enclosing a small, deeply stained dot, the karyosome.

The nucleus is small in relation to the total size of the endameba. This statement is agreed with by practically all observers who have studied the organism, but the measurements given by these observers vary considerably. Von Prowazek stated that the nucleus measured from 1.5 to 4.5 microns in diameter, while Smith and Barrett give the measurement as from 2 to 5 microns in diameter. My experience has been that it is very rare for an individual of this species to show a nucleus exceeding 3.5 microns in diameter and that the average diameter of the nucleus is about 3 microns. The nucleus of this species is considerably smaller than the nuclei of Endamoeba coli and Endamoeba histolytica and very noticeably so in the large vegetative forms of the parasite. The smallness of the nucleus of Endamoeba gingivalis is a very valuable differential point when one is comparing it with other species of endamebae.

The shape of the nucleus is generally circular, but it may be oval or elongated. The nuclear membrane stains black and is well defined in properly prepared specimens, but in those in which differentiation with the iron-alum solution has been carried too far, the membrane may be very dim in outline. The thickness of the membrane also varies with the amount of differentiation — a fact that has caused some confusion in the description of the nucleus, some authorities claiming that the nuclear membrane is thick, others that it is very thin and delicate. If properly differentiated the nuclear membrane of Endamoeba gingivalis is intermediate in thickness between that of Endamoeba coli and that of Endamoeba histolytica, being thinner than that of the former and thicker than that of the latter. In well-prepared specimens the nuclear membrane should appear as a distinct, black circle bounding the nucleus.

The nucleus of this species is very poor in chromatin and in the majority of instances none of this substance can be distinguished within the nucleus, with the exception of the small, black mass, situated centrally, which forms the karyosome. Sometimes, however, the nuclear membrane shows a slight thickening at one or more parts of the periphery, and minute grains of chromatin, stained black, may be observed upon the inner surface of the membrane. In very rare instances a few minute grains may be observed lying between the nuclear membrane and the karyosome, but this is very exceptional. In carefully differentiated specimens a very delicate linin net-work may be observed between the nuclear membrane and the karyosome, imbedded in which there may be one or more dots of chromatin stained dark-brown or black. However, in the majority of instances, the nucleus is composed simply of a well-defined nuclear membrane enclosing a space filled with amorphous, grayish-stained material, at the center of which is a small, deeply stained karyosome.

The general appearance of the nucleus of the vegetative endamebae of this species is very like that of the nucleus of Endamoeba histolytica during the histolytica stage of development. The resemblance is so great, in fact, that Smith and Barrett have brought up the point of the possible identity of the two species; but, in my opinion, the nucleus of Endamoeba gingivalis can be distinguished from the histolytica type of nucleus of Endamoeba histolytica if attention be paid to its smaller size, its thicker nuclear membrane, and its larger karyosome. Of course, individual endamebae occur in which the nucleus is anomalous in some respect, and in such instances a differentiation might be impossible; but, in the vast majority of instances, one should have little difficulty in differentiating the nucleus of Endamoeba gingivalis from that of Endamoeba histolytica, in well-stained preparations. It is also a fact that in no example of Endamoeba gingivalis that I have studied, has a nucleus at all like that of the tetragena type of the nucleus of Endamoeba histolytica been observed, nor have I seen it stated by an observer that such a type of nucleus occurs in this species. Certainly, if the two species were identical, the tetragena type of nucleus would sometimes occur.

The karyosome in this species is small and stains a deep brown or black. It generally consists of a small, compact mass, altho it rarely may appear granular and be composed of several deeply stained grains of chromatin. A centriole has not been observed. In size the karyosome is smaller than that of the nucleus of Endamoeba coli and larger than that of Endamoeba histolytica. If specimens are overdifferentiated in the iron-alum solution, the karyosome may appear to be as small as that of the histolytica type of nucleus of the latter organism, and, in fact, it should be remembered that the appearance of the nucleus of any of the endamebae may be made to resemble that of another

species by variations in the time of staining and the amount of differentiation, so that it is not surprising that observers have differed in their interpretations of the morphologic structure of so minute an object as the nucleus and have formed erroneous conclusions regarding the resemblance of the nucleus of one species to that of another. However, it is true that long practice in the staining of these organisms and the examination of a large amount of material render it possible to differentiate the principal species of endamebae that have been described by their nuclear structure, and Endamoeba gingivalis is no exception to this rule.

The cytoplasm of this species, during the vegetative stage, stains a dull-grayish color and is generally granular in appearance. If the endamebae are small, ingested bodies are not so frequently observed, but, as a general rule, numerous vacuoles are present, many of them containing the debris of ingested bodies, or oval, black bodies, often larger than the nucleus of the endameba, which have been interpreted by most observers as the nuclei of ingested leukocytes. In addition, red blood corpuscles are sometimes seen within the cytoplasm, but these occur much less frequently than one would be led to believe from the usual descriptions of this species.

One of the most characteristic morphologic features of the vegetative stage of development of this species is the very large number of parasites which are filled with oval or round bodies, stained black, and surrounded by a well-marked unstained area. These bodies are evidently contained within vacuoles. They vary in size from 1 micron in diameter to as much as 4 or 5 microns. Altho interpreted by most observers as the nuclei of ingested leukocytes, careful observation will demonstrate that they are not all of the same nature, and that while some are undoubtedly of leukocytic origin, the majority are not derived from these cells, but represent some form of yeast or protozoan organism. Whatever their exact nature, they are generally present in the vast majority of the vegetative forms of Endamoeba gingivalis, and, while not absolutely characteristic of this species—for the same bodies may be observed in both Endamoeba coli and Endamoeba histolytica—they do occur so frequently in this species, and in so many of the organisms, that their presence is of some diagnostic value. It may be stated that I have never observed these bodies within the new species of mouth endameba already referred to as Endamoeba confusa—a fact that helps to distinguish the larger forms of this species from Endamoeba gingivalis.

The vacuoles, with which the cytoplasm of Endamoeba gingivalis is so often filled, vary considerably in size in the individual endamebae, but in the smaller vegetative forms they may be all of about the same size and so numerous that the entire cytoplasm appears to be composed of a net-work of dimly stained material enclosing small, unstained, round or oval areas, the vacuoles. In such instances the vacuoles do not appear to contain ingested material, but in most of the organisms one or more larger vacuoles are present which show ingested bodies.

Reproductive vegetative forms are, in my experience, rarely encountered in stained preparations. I have several times observed endamebae containing 2 nuclei or organisms in which the nucleus was evidently undergoing mitosis, but when compared with the large number of endamebae studied the reproductive forms were singularly rare, even when a very careful search was made for them. This is rather surprising, because in favorable cases the vegetative forms of this species are very numerous and one would expect to find many

undergoing division, but tho such infections were followed from day to day it was the exception to find organisms showing any evidence of division and two-nucleated endamebae were very rare in such massive infections.

When 2 nuclei were observed in a single individual of this species, they were of about the usual size. The nuclear membrane in each case was distinct and the karyosome well stained and situated at the center of the nucleus. The nuclear membrane appeared slightly thinner than it is when only one nucleus is present, and I have never observed any chromatin upon the inner side of the membrane, or free chromatin between the membrane and the karyosome.

A primitive form of mitosis occurs in the nucleus of this species, as the karyosome has been observed to separate into 2 parts connected by a delicate thread of chromatic material; rarely a more or less distinct nuclear spindle may be demonstrated. After the division of the karyosome the nucleus becomes elongated and a constriction occurs near the center, which increases until the nucleus is divided into 2 portions of equal or nearly equal size. Various stages of this process may be traced in stained preparations but only with difficulty, owing to the relative infrequency of dividing forms.

I have several times observed individuals of this species which have been interpreted by some authorities as examples of reproduction by budding or gemmation, but it is my belief that this process is a degenerative one in this species, just as it is in Endamoeba histolytica. Organisms are observed in stained preparations which contain granules, rods, and clumps of chromatic material within the cytoplasm, while the nucleus has either disappeared or appears to be breaking up and supplying this material to the cytoplasm. Sometimes the deeply stained chromatic material appears to be collected near the periphery of the parasite and small projections containing some of the same material may be seen apparently being budded from the periphery, but the morphologic details are exactly the same as those observed in the so-called budding forms of Endamoeba histolytica and there is no more reason to believe that this is a reproductive process in Endamoeba gingivalis than that it is in Endamoeba histolytica, where it has been proved to be degenerative in character.

The Pre-Cystic Stage.—The pre-cystic forms of this species, in stained preparations, are characterized by their small size, homogeneous cytoplasm, and the larger size of the nucleus in comparison with the total size of the parasite. These forms have not been described before, evidently having been mistaken for vegetative forms. They do not occur very frequently and are rare even in those infections which present them.

The pre-cystic stained forms seldom measure more than 10 microns in diameter; the cytoplasm is homogeneous in appearance, finely granular in structure, and stains a brownish gray or distinct gray with the method described. No vacuoles are present, in most instances, and the cytoplasm is free from ingested bodies. The nucleus is large in relation to the total size of the parasite, generally measuring over 4 microns in diameter, and frequently as much as 5 or 6 microns. The nuclear membrane is well defined, staining a dark brown or black, and is slightly thicker than it is in the vegetative forms. The karyosome stains black, is situated at the center of the nucleus, and is larger than it usually is in the vegetative forms. The space between the karyosome and the nuclear membrane stains a dim gray and is generally free from chromatin, altho in some instances a few small, black granules may be seen in this space or upon the inner side of the nuclear membrane. At this stage of development there is no sign of a cyst wall. I have never observed any evidence of reproduction in these pre-cystic forms and it is my belief that they are merely individuals that are about to encyst.

The Cystic Stage.—The cystic stage of Endamoeba gingivalis was first mention by Chiavaro,[7] but he does not describe the cysts and does not state whether this is a reproductive process in this species, altho he pictures a cyst containing 2 nuclei. It must be said that his two drawings of the cystic forms of this species are not at all convincing and it is most unfortunate that no description of the morphology of the cysts is given in his paper. The only other authors to mention cysts in this species are Smith and Barrett,[14] who state that they have observed "dauer cysts" but no reproduction cysts, and no description is given in their article of the "dauer cysts."

In my experience the cystic forms of this species are very rarely observed, the conditions in the mouth apparently being most favorable to the development of the vegetative forms. I have also found that, when present, they occur in very small numbers, the vegetative forms, even in such cases, far out-numbering them.

The cysts measure about 10 microns in diameter, but examples are observed which measure as much as 12 microns. The cytoplasm appears homogeneous and finely granular and stains a dark brown or brownish gray with the method described. Vacuoles and ingested bodies are absent, as in the pre-cystic forms; in rare instances I have observed cysts containing a single, large vacuole, but when fully developed the cysts are free from vacuoles. The nucleus is well defined, generally situated at or near the center of the cyst, altho it may be placed at the periphery. It varies in size but generally measures 3 to 4 microns in diameter, most of the cysts showing a nucleus smaller than that observed in the pre-cystic forms. The nuclear membrane stains a deep black and seldom presents any chromatin dots on its inner surface, being perfectly smooth and slightly over a line in thickness. The karyosome is composed of a single, small mass of black-stained chromatin, situated at or near the center of the nucleus, while the space between the karyosome and the nuclear membrane contains no chromatin, appears finely granular, and stains a light gray in color.

The cyst membrane varies in appearance apparently with the age of the cyst. At first it is very difficult to differentiate this membrane from the periphery of the cytoplasm, and at this stage of development it is often impossible to tell whether one is dealing with a pre-cystic or cystic form. The first appearance of a membrane consists in a very delicate line surrounding the periphery of the organism; in older cysts this line appears thicker and in some instances a definite double-contoured membrane may be easily distinguished. In my experience the cyst membrane is generally more delicate and more difficult to distinguish in this species than it is in either Endamoeba coli or Endamoeba histolytica.

Reproductive changes, so far as I have been able to observe, do not occur in the cysts of Endamoeba gingivalis, for I have never observed a cyst with more than one nucleus, nor have I seen any changes in the nucleus that could be interpreted as reproductive in character. So far as the evidence goes, cyst formation in this species is purely protective in character, for no investigator has yet given any proof that multiplication of the parasite occurs within the cyst. From the morphology of the cysts observed in stained preparations it is evident that before encystment the endameba frees itself of all ingested material and that the cytoplasm becomes more dense in structure, as it stains much more intensely than does the cytoplasm of the vegetative forms. These phe-

[14] Jour. Parasitol., 1915, 1, p. 167.

nomena are common to all endamebae before encystment, so that this species does not differ, in this respect, from other species described. It may be that further research will demonstrate that reproduction occurs with the cysts, but at the present time there is no evidence that encystment is anything more than protective in nature.

THE IDENTITY OF ENDAMOEBA GINGIVALIS AND ENDAMOEBA HISTOLYTICA

Because of the statements of Smith and Barrett[15] that they were unable to differentiate Endamoeba gingivalis morphologically from Endamoeba histolytica it is necessary to consider briefly this phase of the subject. They state that microscopically they were unable to differentiate the two species, while both reproduce by binary fission, and both fail to produce reproduction cysts. I am unable to agree with these authors as to the morphologic resemblance of Endamoeba gingivalis to Endamoeba histolytica and with their statement regarding reproduction by gemmation in the two species and the absence of reproductive cysts in Endamoeba histolytica.

Endamoeba gingivalis resembles Endamoeba histolytica morphologically only when the latter is in that portion of its life cycle generally known as the histolytica stage. At this time the nucleus of Endamoeba histolytica does resemble, at first glance, the nucleus of Endamoeba gingivalis, but it is much larger in comparison with the total size of the organism, while the nuclear membrane is more delicate and the karyosome considerably smaller. The size of Endamoeba gingivalis averages much smaller than that of Endamoeba histolytica and its motility is much more restricted and less active. The ectoplasm of Endamoeba gingivalis, in living specimens, is never as refractive and glass-like as that of Endamoeba histolytica and its pseudopodia are much smaller.

The form of reproduction by "gemmation" mentioned by Smith and Barrett, has now been definitely proved to be a degenerative process, having nothing whatever to do with reproduction, and the fact that it occurs in this species, as well as in Endamoeba histolytica, simply demonstrates that both species undergo the same form of degeneration.

As is well known, at one stage of its development Endamoeba histolytica presents a very definite type of nucleus, known as the tetragena type; but this type of nucleus has never been observed in Endamoeba gingivalis, no matter what the clinical character of the infection. If the species were identical, such a type of nucleus would most certainly

[15] Jour. Parasitol., 1915, 1, p. 173.

have been observed; for there is no reason to believe that this stage in
the development of the species would not occur in the mouth.

The statement of Smith and Barrett that neither species develops
reproductive cysts is erroneous so far as Endamoeba histolytica is con-
cerned, for the well-known four-nucleated cysts of this species are
relied upon to distinguish this stage in the development of the organism
from the cysts of Endamoeba coli, which contain 8 or more nuclei. The
fact that the cysts produced by Endamoeba gingivalis have only one
nucleus and are therefore probably not reproductive in character, alone
serves to distinguish this species from Endamoeba histolytica.

If we add to these differences in morphology and life-cycle the fact
that neither rectal injection nor feeding of material containing Enda-
moeba gingivalis gives rise to diarrhea or dysentery in animals, in
which such symptoms are readily produced by Endamoeba histolytica, I
believe that the evidence is conclusive that the two species are not
identical.

RELATION OF ENDAMOEBA GINGIVALIS TO DISEASE

Altho the assertion that this endameba is the cause of certain dis-
eases of the teeth and gums has been made by numerous authorities,
some of whom have even endeavored to trace a relation between Enda-
moeba gingivalis and certain systemic conditions, it may be stated that
at the present time the question is far from settled tho there is a
constantly growing opinion among those who have had the largest
experience with pyorrhea alveolaris and other diseases of the mouth,
that Endamoeba gingivalis has little, if anything, to do with the con-
ditions present, being merely a harmless commensal, as is Endamoeba
coli in the intestine.

Despite the previous paper by Lewald,[5] published in 1907, and
apparently unknown to Barrett, in which he demonstrated that the
species of endameba under discussion could be found, upon careful
search, in the mouths of most healthy persons, 71 positive results
having been obtained by him in 100 such individuals, Barrett,[6] in con-
junction with Smith, published a paper in 1914 in which they asserted
that this parasite was probably the cause of pyorrhea alveolaris, basing
their conclusions upon the presence of the organism in the lesions of
the disease and on the improvement of the condition after the adminis-
tration of emetin, a well-known amebacide. While the paper of Barrett
was conservative, the same cannot be said of the paper by Bass and

Johns,[9] published in 1915, in which these authors state, unconditionally, that pyorrhea is due to endamebae, altho they give no scientific evidence for the statement beyond the finding of the endamebae in the lesions of the disease and the improvement of the condition after the administration of emetin. While the conclusions drawn in these two papers cannot be said to have been proved, they have served to incite research in this direction, being responsible for most of the work by both dentists and physicians upon this organism during the past two years.

Shortly after the publication of Barrett's original paper, Angelo Chiavaro,[7] working in Grassi's laboratory in Rome, published the results of his researches upon Endamoeba gingivalis and its relation to pyorrhea. He found the endameba present not only in the pus of cases of pyorrhea, but also in the cavities of carious teeth and in the materia alba and other deposits upon sound teeth. In his conclusions he states that while this species of endameba is found in the pus of most cases of pyorrhea alveolaris it is also generally present in the materia alba of the sound teeth if they are not kept in hygienic condition, and in carious cavities if the reaction is acid. He states, in conclusion: "The endamoeba has not a pathogenic action; on the contrary, as it feeds on bacteria, it is most probably an aid to the autodisinfection of the mouth."

That this species of endameba is found in a very large percentage of healthy mouths, as first shown by Lewald, is demonstrated by the researches of Anna Williams,[16] who found it in 30% of children with healthy gums. She also found this parasite in 50% of children with healthy gums and carious teeth; in 84% of children showing tartar around the teeth and receding gums; and in 94% of children with spongy and bleeding gums. She makes the following statement:

We can say nothing definite yet as to the significance of the amebas in these mouths. Finding them so often in apparently healthy mouths, and in such young children, does not agree with the statements of Bass and Johns, and Barrett, that they are not found in healthy mouths.

I have repeatedly found *Endamoeba gingivalis* in the materia alba around perfectly sound teeth and in perfectly healthy mouths, and have failed to find the parasite in some typical cases of pyorrhea, and while this parasite undoubtedly occurs more frequently, or, at least, is more easily demonstrated when the gums are diseased, its mere presence is certainly no proof of its etiological relationship to pyorrhea alveolaris or other diseases of the mouth.

[16] N. Y. Path. Soc., March, 1915. Cited by Merritt, New York Med. Jour., 1915, 102, p. 281.

Since the publication of the original paper of Barrett, the treatment of pyorrhea with emetin, based upon the supposed causation of the disease by endamebae, has been vigorously pursued by the dental profession throughout the country, and it must be admitted that the reports regarding its efficiency are most conflicting and the more recent articles most disappointing regarding the value of the drug as a specific for this disease. Such dental authorities as Henrici and Hartzell,[10] Sarrazin,[11] and Merritt[17] believe that the emetin treatment of pyorrhea is not curative and that there is no evidence sufficient to prove that the disease is due to endamebae. Hartzell states that, in his hands, emetin has failed even to benefit the condition, while Sarrazin saw no decided improvement in the patients under his observation. Merritt is inclined to believe that what benefit does follow the use of emetin in pyorrhea is due to the fact that it is a hemostatic and concludes: "Whatever its action may be, there is at present no trustworthy evidence that it will cure pyorrhea."

Through the kindness of Dental Surgeon Minot E. Scott, U. S. Army, I have had the opportunity of following several patients suffering from pyorrhea alveolaris who were being treated with emetin, and I have found that while the endamebae generally decrease in number during its administration, careful search will show motile and apparently healthy endamebae in the lesions throughout the period of administration. In one instance there was no appreciable decrease in their number altho the drug was exhibited in the most approved manner for several weeks.

It should also be remembered that emetin is not without action upon other organisms, as has been shown by Vedder,[18] so that all the improvement following its use in pyorrhea cannot logically be credited to its amebacidal action, especially when the endamebae do not disappear tho the symptoms improve, as has happened in some instances. In all these cases I have found that spirochetes are present in immense number and it is just as reasonable to believe that these cause pyorrhea and that the emetin has some action on them (as they certainly lessen in number under treatment) as it is to claim that endamebae are the cause of the disease because of their presence before, and decrease after, the administration of this drug.

Price and LaRue,[12] in a summary of the present status of the emetin treatment of pyorrhea, prepared for the Scientific Foundation

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[17] New York Med. Jour., 1915, 102, p. 279.
[18] Bull. Manila Med. Soc., 1911, 3, p. 48.

and Research Commission of the National Dental Association, after considering the arguments for and against the theory of the causal relationship of Endamoeba gingivalis to pyorrhea alveolaris, state that they have observed many cases of severe typical pyorrhea in which this endameba could not be demonstrated, and that in many cases of the disease emetin gives very poor or no results. In their opinion the proof is yet insufficient that these organisms have anything whatever to do with the production of the disease.

At the present time the only proof we possess of the etiologic relationship of Endamoeba gingivalis to pyorrhea alveolaris consists in its almost constant presence in the lesions of the disease and in the fact that emetin, properly administered, greatly benefits a large majority of the patients suffering from the disease. There is absolutely no experimental proof of the etiologic relationship of this parasite to pyorrhea, while against this relationship we have the following facts: The occurrence of the parasite in a large percentage of healthy mouths and in the material that can be scraped from healthy teeth and gums; the occurrence and persistence of the parasite in patients treated with emetin, even when marked improvement in the clinical symptoms has occurred; the absence of the parasite in some typical cases of pyorrhea; the lack of improvement with emetin shown in numerous instances of the disease, altho the endamebae may disappear; and the fact that emetin acts upon other organisms as well as upon endamebae and the possibility that the improvement that often follows its administration may be due to such action or to a favorable action upon the tissue cells.

In conclusion, it may be stated that if one considers carefully the data that have accumulated upon this subject there is but one deduction which can be drawn, and that is that it is more than doubtful that Endamoeba gingivalis is the cause of pyorrhea alveolaris, and that it is yet too early to make positive statements regarding the relationship of this parasite to disease.

EXPLANATION OF PLATE 6

Fig. 1. Young form of Endamoeba gingivalis. Note size of nucleus, and homogeneous cytoplasm, which contains some bacteria.

Figs. 2, 3, and 4. Vegetative forms of Endamoeba gingivalis, showing the ingested bodies so frequently observed. Note the size of the nucleus in relation to the total size of the endameba.

Fig. 5. Dividing form of Endamoeba gingivalis, containing ingested bodies and bacteria. Note minute size of the two nuclei.

Fig. 6. Dividing form of Endamoeba gingivalis with unusually large nuclei. In this organism the nuclei closely resemble the nucleus of Endamoeba coli.

Figs. 7 and 8. Vegetative forms of Endamoeba gingivalis. In Fig. 7 the nucleus is very typical. In Fig. 8 the nuclear membrane is thickened at two portions of the periphery.

Figs. 9 and 10. Pre-cystic forms of Endamoeba gingivalis. Note larger size of the nucleus in these pre-cystic forms and the homogeneous cytoplasm.

Fig. 11. Cystic form of Endamoeba gingivalis showing single outline to the cystic membrane. Note smaller size of the nucleus and homogeneous, deeply stained cytoplasm.

Fig. 12. Cystic form of Endamoeba gingivalis, showing double-contoured membrane.

PLATE 6
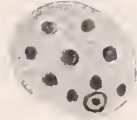
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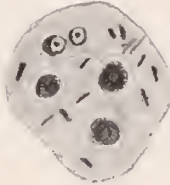
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THE EXAMINATION OF THE URINE AND FECES OF SUSPECT TYPHOID-CARRIERS WITH A REPORT ON ELATERIN CATHARSIS *

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It is our purpose to present a brief study of the results of examination of 290 specimens of urine and 298 specimens of feces, and to discuss certain practical phases of the work in connection with the search for typhoid-carriers.

ELATERIN CATHARSIS

That many typhoid-carriers pass the typhoid bacillus intermittently has long been recognized, and it has been the common experience of bacteriologists that feces examinations are frequently negative even in known carriers. Hence, the opinion prevails that several specimens, collected over a period of considerable time, should be found negative before a suspect can be definitely discharged as negative, or a typhoid-carrier or convalescent pronounced definitely cured.

In a recent article by Dryer, Walker, and Gibson[1] particular attention is given to an improved method of technic, whereby a larger quantity of fecal material can be examined, thereby increasing the chances of securing positive results in cases in which the bacilli are sparse in number. The authors mentioned make use of actinic light to inhibit the growth of organisms other than typhoid and paratyphoid bacilli.

On the basis of the fact that postmortem examinations of typhoid patients commonly demonstrate the presence of typhoid bacilli in largest numbers in the small intestine, whereas they are seldom found in the colon, a plan was evolved in this laboratory of using, in carrier cases, a suitable cathartic to bring down the contents of the small intestine. The selection of such a cathartic involves two essential considerations: (1) The hydrogogue cathartic selected, must be sufficiently powerful to empty the bowels thoroughly and to bring down the contents of the small intestines, and (2) it must be devoid of antiseptic properties.

Elaterin appeared to meet both these requirements satisfactorily. It

[1] Lancet, 1915, 1, p. 324.

was tried out experimentally, and its use adopted as routine procedure in the examination of typhoid-carriers in this laboratory. The dosage ordinarily given varies from 0.1 to 0.2 of a grain of elaterin. Given in the evening, it usually results in a copious bowel movement on the following morning. The first portion of the bowel movement, consisting of formed stool, is discarded. The last or liquid portion is retained for examination.

Our first experience in the use of elaterin was in connection with a restaurant epidemic, for which a waitress who proved to be a typhoid-carrier was responsible.   In this case 3 successive specimens of
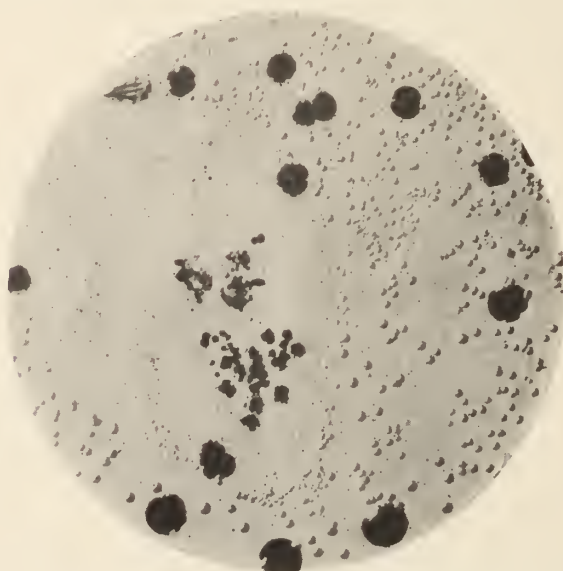
Fig. 1.   Portion of an Endo plate inoculated with feces of a typhoid-carrier after elaterin catharsis, showing numeric relation between typhoid and colon colonies.   Typhoid colonies are colorless; colon colonies are dark-red.   The ratio of typhoid to colon colonies in this specimen was 31 to 1.

feces collected without the use of elaterin, were negative.   Elaterin was administered prior to the collection of the fourth specimen, with the result that typhoid bacilli were isolated without difficulty in large numbers.   In a second similar case, 2 negative specimens were obtained prior to the administration of elaterin, while the third specimen, obtained by the use of elaterin, yielded large numbers of typhoid bacilli.   The first of these patients continued to yield positive elaterin stools for a number of weeks, until she was finally lost sight of.

The second one continued to yield positive elaterin stools for a period of three months, after which, under suitable treatment, the stools became negative. Since the adoption of the use of elaterin catharsis as a routine procedure in the examination of suspect carriers, 3 typhoid-carriers have been detected successively by examination of the first stool presented for test.

In one case only, which we shall designate as Carrier A, have we been able to make a detailed study of stools collected both with and without elaterin. The findings, however, with reference to the numeric relation existing between typhoid and colon colonies are interesting. Table 1 shows the approximate quantitative relationship between the two organisms with and without the use of a cathartic. These figures do not represent the bacteria content of any specified quantity of feces, but they indicate the ratio of typhoid colonies to colon colonies which existed on the plates selected as most suitable for counting. Up to the present time we have examined 23 stools from Carrier A. Nine specimens were collected by the use of elaterin, 13 without a cathartic, and 1 by the use of magnesium sulfate. Typhoid bacilli were isolated from all except one of the specimens obtained from this patient. This specimen was collected without elaterin.

TABLE 1

The Ratio Between Typhoid and Colon Bacilli in Carrier A

Using Elaterin			Using No Cathartic			Using Magnesium Sulfate		
Typhoid	:	Colon	Typhoid	:	Colon	Typhoid	:	Colon
31	:	1	1	:	2	4	:	1
27	:	1	33	:	1			
125	:	1	1	:	1			
110	:	1	15	:	1			
156	:	1	18	:	1			
67	:	1	22	:	1			
41	:	1	5	:	1			
29	:	1	1	:	200			
3	:	1	1	:	100			
			1	:	300			
			0	:	500			
			1	:	150			
Total... 589	:	9	99	:	1,258	4	:	1
Average 65.4	:	1	1	:	12.7	4	:	1

It will be noted in Table 1 that the typhoid-colon ratio consistently favors the use of elaterin, the average with elaterin being 65.4 typhoid to 1 colon, and without elaterin, 1 typhoid to 12.7 colon. As judged by the typhoid-colon ratio, typhoid bacilli were 830.6 times more numerous in elaterin stools than in normal stools.

Figure 1 is representative of the appearance of the Endo plates made in the case of Carrier A from elaterin stools.

The findings thus far obtained, appear to justify the use of elaterin as a valuable adjunct to the technic of feces examination.

Technic

Materials Necessary.—Endo's medium, Petri dishes, capillary pipets, and glass rods.

Endo's medium is prepared as described by Kenyoun and Deiter.[2] The agar is stored in 100-c.c. portions in bottles until immediately before use. When required the agar is melted in an Arnold sterilizer, and sodium carbonate or acid, as determined by the titration, is added. In our experience, it is usually necessary to add from 0.5 to 0.9 c.c. of a 2.5% solution of anhydrous sodium carbonate. To all bottles we add, in addition to the sodium carbonate, 1 gram of crystallized lactose C. P., 5 c.c. of a 10% solution of crystallized sodium sulfite, and 0.7 to 0.9 c.c. of a half-saturated alcoholic solution of basic fuchsin. Seven or eight plates can usually be poured from one 100-c.c. bottle. After the plates have hardened they are ready for use.

The capillary pipets are made from glass tubing about 8 mm. in diameter. The capillary opening is about 2 mm. in diameter. A non-perforated rubber nipple supplies the needed suction.

The glass rods are bent at right angles, about 2 cm. from one end, into the form of an "L."

The glass rods and pipets are sterilized by boiling for 5 minutes before and after use.

Plating of Specimens.—When urine is examined, the specimen is centrifugated and the sediment used. If a hard specimen of feces must be accepted, an emulsion is made with sterile salt solution. Liquid feces are used without dilution. In examining urines 2 or 3 plates are necessary, and in examining feces 5 plates are necessary.

A capillary drop of liquid feces is placed on the first plate and thoroughly spread over the plate with the short arm of the L-shaped rod. The glass rod is then rubbed over each of the other plates in succession without further addition of feces. This process usually gives numerous discrete colonies on the last two or three plates. A control plate should invariably be inoculated with known typhoid and colon bacilli upon opposite halves of the plate. The plates are then incubated for 24 hours at 37 C. Suspicious typhoid-like colonies are picked off and planted in tubes containing 10 c.c. of plain broth, made according to the following formula: Peptone—2 gm., meat extract—1.5 gm., salt—0.5 gm., water—100 c.c.

It is our practice to pick 10 colonies, if the number of suspicious colonies is 10 or more. In case fewer than 10 suspicious colonies are present, the entire number are picked. If no suspicious colonies are present, the test is discontinued at this point as negative. A known culture of typhoid bacilli is at the same time planted in several broth tubes as a control.

After an incubation of from 12 to 24 hours at 37 C., the cultures are examined. Tubes showing (1) scum or pellicle, and (2) heavy precipitate at bottom, are discarded. The typhoid bacillus is light and moves quickly on shaking (compare with control).

[2] Am. Jour. Pub. Health, 1912, 2 (O. S., 8), p. 979.

Macroscopic Agglutination Test with Typhoid Bacilli.—One capillary drop of antityphoid serum of about 1 : 1,000 titer is added to each tube. The tubes are placed in the incubator at 37 C. for 15 minutes. If no agglutination occurs in the control tube in this time, another drop is added to all the tubes and so on until a typical agglutination occurs in the control tube. If typical agglutination, comparable with that of the control, occurs in other than the control tubes, the presence of typhoid bacilli is indicated. The macroscopic agglutination test is then confirmed by removing a loopful of the agglutinated culture and examining it microscopically upon a hanging-drop slide.

Diagnostic Points.—Positive macroscopic and microscopic agglutination tests with typhoid bacilli are sufficient basis for the detention of the suspect carrier.

Confirmatory Tests : (1) The organism must be motile in young broth culture. (2) It must be a bacillus and must be gram-negative. (3) It produces typical dew-drop colonies on Endo's medium. (4) In litmus milk it produces slight alkalinity in 24 hours, and slight acidity after 48 hours, with coagulation in about one week. (5) In dextrose broth it produces acidity but no gas. (6) In lactose broth it produces no acidity or slight acidity without gas. (7) On Russell's medium it grows with unchanged surface and red color in the deep portions. (8) In Dunham's peptone it produces no indol.

Prior to the examination of feces and urine, it is usually advisable to make agglutination tests of the blood with typhoid bacilli. Cases showing positive reactions, as well as those giving suspicious typhoid histories, should be tested repeatedly for typhoid bacilli in the urine and feces before being discharged as negative.

Tests for typhoid bacilli in urine should be preceded by a chemical test for formaldehyd, which appears in urine as a result of the administration of hexamethylenamin, and which effectually prevents the isolation of typhoid bacilli from the urine.

THE EFFECT OF LACTOSE BILE "ENRICHMENT"

The use of lactose peptone bile as an enrichment medium for the detection of colon bacilli in water and sewage was first recommended by Jackson.[3] It has since been demonstrated that its effect is an inhibition rather than an acceleration of the growth of the organism. Its value depends on the fact that it probably inhibits the growth of other organisms more strongly than it does that of the colon bacillus, its inhibition of the colon bacillus being probably not sufficient to prevent the latter's detection.

The use of lactose bile medium has also been recommended in the examination of water supplies for typhoid bacilli. We tried out this medium over a period of 2 years, in connection with our routine examination of feces and urine for typhoid bacilli.

The 290 specimens of urine and the 298 specimens of feces here reported were plated directly on Endo's medium in the usual way. A second portion of each specimen was inoculated into lactose peptone bile, consisting of ox

[3] Jour. Infect. Dis., 1907, Suppl. 3, p. 30.

bile containing 1% of peptone and 1% of lactose. The bile inoculations were grown for 48 hours at 37 C., and then material from a portion of the bile culture was inoculated upon Endo's medium, by technic similar to that of the direct inoculations.

Table 2 shows definitely the inhibiting effect of the bile medium on the typhoid bacillus. In fact, in only one case, a urine examination, was the organism isolated from the bile when the direct plate was negative. This, in view of the other findings, we are inclined to attribute to an error in the technic of the direct examination. In the case of 8 specimens of urine and 38 specimens of feces, the organism isolated from the direct plates was lost in passage through the lactose bile. In only 2 specimens of feces and 1 of urine were the organisms isolated both from the bile medium and the direct plates. The total number of specimens of urine and feces found positive on direct plating was 50. The total number of specimens of urine and feces found positive after bile enrichment was 5.

With reference to the influence of a soft stool upon the positive result, it will be seen from the table that 21 elaterin stools were positive and 7 naturally soft stools were positive, whereas only 2 hard stools were positive. These figures added to our later results give totals as follows:

Elaterin stools positive............................35
Soft stools without elaterin positive.................18
Hard stools positive 4

As is evident from the quantitative results of the platings, typhoid bacilli are commonly much more numerous in soft, or diarrheal, stools than in formed stools. Therefore, a carrier is probably most infectious while he is in a diarrheal condition, and least infectious, or possibly not at all infectious, in the absence of diarrhea. This circumstance offers at least a partial explanation of the intermittent infectivity of many carriers.

With reference to the effect of hexamethylenamin, administered by mouth, upon the urinary typhoid-carriers, we may add that we have not been able to demonstrate typhoid bacilli in a specimen of urine which gave a reaction for formalin, altho we have examined at least one urinary carrier in our series.

SUMMARY AND CONCLUSIONS

The use of elaterin catharsis is of material assistance in the examination of the feces of suspect typhoid-carriers.

TABLE 2

RESULTS OF EXAMINATION OF URINE AND FECES FOR TYPHOID BACILLI WITH AND WITHOUT
LACTOSE BILE ENRICHMENT

Specimens Positive by One or Both Methods. Specimen Number	First Portion, Plated Directly on Endo Medium		Second Portion, Grown in Lactose Bile 48 Hours and Plated on Endo Medium		Remarks
	Urine	Feces	Urine	Feces	
66	+	—	..	..	
160	+	—	+	..	
698	..	..	..	+	
722	+	—	—	—	
724	—	+	—	—	Elaterin
784	—	+	—	—	Elaterin
795	—	+	—	—	Elaterin
799	—	+	—	—	Elaterin
801	—	+	—	—	Elaterin
807	—	+	—	—	Elaterin
819	—	+	—	—	Elaterin
820	+	+	—	—	Elaterin
827	—	+	—	—	
828	—	+	—	—	Elaterin
830	+	+	—	—	
841	—	+	—	—	Elaterin
846	—	+	—	—	
849	+	+	—	—	Elaterin
855	+	—	—	—	
878	—	+	—	—	Elaterin
879	+	+	—	—	
900	—	+	—	—	
901	..	+	..	—	Elaterin
904	—	+	—	—	Elaterin
911	—	+	—	—	Soft
917	—	+	—	—	Soft
3	—	+	—	—	Soft
11	—	+	—	—	Elaterin
19	—	+	—	—	Hard
23	—	+	—	—	Elaterin
37	—	+	—	—	
46	—	+	—	+	
47	—	+	—	—	Soft
58	—	+	—	—	
67	—	+	—	—	Elaterin
82	—	+	—	—	
91	—	+	—	—	Elaterin
105	+	+	—	—	Soft
116	+	+	—	+	Soft
127	—	+	—	—	Elaterin
135	—	+	—	—	Elaterin
148	—	+	+	—	Soft
162	—	+	—	—	Elaterin
189	—	+	—	—	
657	—	+	—	—	
Total negative by both methods....	244*	241	244	241	
Totals (a) Positive......	10	40 {21 elaterin, 7 soft...., 2 hard...}	2	3	
(b) Negative.....	277	246	284	282	
Total number of specimens........	287	286	286	285	

* Formaldehyde present in 5

The value of a negative finding in the feces of a given typhoid-carrier suspect is enhanced by the use of elaterin.

The use of Endo plates, prepared according to the method of Kenyoun and Deiter, constitutes, in our experience, the most satisfactory practical technic for the examination of urine and feces for typhoid bacilli.

The effect of lactose peptone bile "enrichment," when used in the attempt to isolate typhoid bacilli from urine and feces, is that of inhibition of the growth of the typhoid bacillus. As a rule, typhoid organisms that are demonstrable by direct plating are lost by passage through lactose bile.

A diarrheal state on the part of the suspect greatly increases the chances of isolating the typhoid bacillus from the stool.

The infectivity of typhoid-carriers in whom the organism is carried by the feces, is greatly enhanced by the presence of a diarrheal condition, and is in all probability small, or possibly negligible, in the absence of diarrhea.

Our experience with the administration of hexamethylenamin to urinary typhoid-carriers indicates that typhoid bacilli are not demonstrable in the urine after recent administration of this drug.

THE BACTERICIDAL AND PROTOZOACIDAL ACTIVITY OF EMETIN HYDROCHLORID IN VITRO *

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While there are in the medical and dental professions differences of opinion regarding the rôle of Endamoeba gingivalis, Gros, in the etiology of pyorrhea alveolaris and the efficacy of emetin in the treatment of this disease, as originally discovered and announced by Smith and Barrett[1] and independently by Bass and Johns,[2] and confirmed by numerous investigations by Smith, Middleton, and Barrett,[3] Evans and Middleton,[4] Chaplin,[5] and others, experimental investigations, especially those of Vedder[6] and Wherry,[7] have shown conclusively the high amebacidal action in vitro of ipecac and its chief alkaloid emetin.

Investigation of the probable bactericidal action of ipecac and of the alkaloid emetin has been incomplete. It is well known that various bacteria, including streptococci, pneumococci, various bacilli, spirochetes, leptothrices, etc., may be found in abundance in pus from the gums of persons suffering with pyorrhea alveolaris, and most observers, including those who advocate emetin in the treatment of this disease, assign to these bacterial species some rôle, usually a collateral one, in the etiology of this disease. Smith and Barrett[8] incline to the belief that the ameba act "in symbiotic relation with some or all of the vegetable organisms with which they are in association in nature; that by their proteolytic power they prepare a highly fitting pabulum for the growth of bacteria in the form of end products of their digestion of leukocytes, red blood cells and perhaps fixed cells as well, and thus favor a rank mycotic growth about them; that by their ingestion and destruction of these bacteria they set free a not inconsiderable amount of bacterial toxins of different kinds and of varying influences; and that these toxins are locally necrosing and of essential importance in determining and maintaining the gingival and alveolar inflammation, and too may be diffused and be productive of a widespread series of complications in the body of the host, commonly discussed in connection with the 'oral sepsis' of Hunter." Bass and Johns,[2] taking a more decided view of the rôle of the amebae in the pathogenesis of Rigg's disease, regard them as the specific cause.

[1] Dental Cosmos, 1914, 56, p. 948.

[2] New Orleans Med. and Surg. Jour., 1914, 68, p. 456. Jour. Am. Med. Assn., 1915, 64, p. 553.

[3] Jour. Am. Med. Assn., 1914, 63, p. 1746.

[4] Ibid., 1915, 64, p. 422.

[5] Dental Cosmos, 1915, 57, p. 189.

[6] Jour. Am. Med. Assn., 1914, 62, p. 501. Bull. Manila Med. Soc., 1911.

[7] Jour. Infect. Dis., 1912, 10, p. 162.

[8] Oral Health, 1915, 5, p. 137.

In view of the fact that the associated micro-organisms disappear from the pockets of disease with the cessation of suppuration, tho not as completely as do the amebae, and that temporary improvement of the lesions is frequently obtained and occasionally a permanent cure effected by cleansing and treatment with various antiseptics and by bacterial vaccine therapy, we have investigated in more detail the probable bactericidal properties of emetin, with the hope of shedding more light on the action of this drug in the treatment of pyorrhea alveolaris, either by showing that the drug has a combined amebacidal and bactericidal action, or, in case the latter were absent or so feeble as to be without commensurate influence, to add thereby to the evidence indicating the intimate rôle of amebae in the pathogenesis of pyorrhea alveolaris and the efficiency of emetin in its treatment.

SCOPE OF THE INVESTIGATION

We have studied the bactericidal action of emetin hydrochlorid in vitro, in fluid and solid culture media, on the following micro-organisms: Staphylococcus aureus, Streptococcus salivarius, B. typhosus, B. anthracis, and B. subtilis. These bacteria were selected as test organisms mainly because their hardy character and their ability to grow uniformly in ordinary culture media make them adaptable for germicidal studies. The bacillus of anthrax was included because of the time-honored and empirical custom in the Philadelphia Hospital for Contagious Diseases of treating the wound of anthrax, after excision of the lesion, with powdered ipecac; one of us (Kolmer) has observed that in a number of cases so treated the drug may have been responsible for the destruction of anthrax bacilli in the wound. As will be shown later in this report, emetin hydrochlorid was found in our experiments to possess a relatively high bactericidal power for the spore-bearing anthrax bacillus, and in order further to study this action Bacillus subtilis was included in the list of test organisms. We have included also a study of the trypanocidal action of this alkaloid in vitro on Trypanosoma lewisi and Trypanosoma equiperdum, as well as on Endamoeba gingivalis, Gros.

In view of the results of this study, showing that emetin hydrochlorid possesses some bactericidal action, and in view of the beneficial results reported by Bass and Johns and others in the treatment of pyorrhea alveolaris and various complications, especially arthritic conditions (Evans and Middleton), with the hypodermatic administra-

tion of emetin, we have also studied the bactericidal action of emetin in vivo, the results of this study being given in a separate communication.

In the studies here reported, we have used dilutions of pure phenol as control germicide, so that the bactericidal value of emetin in the various methods employed and with the different test micro-organisms is expressed in terms of comparison with phenol.

BACTERICIDAL ACTION OF EMETIN HYDROCHLORID

In 1910 Vedder[9] reported that ipecac possessed no specific bactericidal effect against B. dysenteriae, notwithstanding that his studies clearly indicate the antiseptic, or even bactericidal, value—tho of undetermined degree—of ipecac in the case of the Shiga and Flexner strains of B. dysenteriae, B. typhosus and B. paratyphosus, and Staphylococcus aureus. Hitchens[10] found that a 4% solution of emetin failed to kill B. typhosus in 15 minutes. Frazier[11] found that a dilution of 1 : 25,000 in serum agar possessed antiseptic and even germicidal power for B. typhosus. Wherry[7] found emetin in solution of 1 : 20,000 bactericidal in 48 hours against the symbiotic bacillus in the ameba culture with which he worked.

In all our work tablets of emetin hydrochlorid (⅓ grain = 0.0215 gm.) for hypodermatic use, were used. Various dilutions (4, 2, 1, 0.5, and 0.25% solutions) in plain sterile neutral broth were freshly prepared for each experiment.

In all experiments the test micro-organism was grown for 24 hours in plain neutral broth and filtered through sterile filter paper before being used. Streptococcus salivarius was cultivated in serum broth. The cultures of B. anthrax and B. subtilis were briefly shaken with glass beads before filtration in order to break up chains of bacilli. In all instances the filtered cultures were slightly less dense than before filtration, but filtration was considered necessary in order to remove clumps of bacteria; it in no way interfered with the experiments.

In all experiments corresponding dilutions of pure phenol in sterile distilled water were employed. Likewise, each experiment had several culture controls to assure us that the culture was viable, able to multiply in the media used, and that the dose was sufficient.

With Rideal-Walker and Hygienic Laboratory Methods.[12]—Several experiments were conducted according to these methods with dilutions of emetin controlled by dilutions of phenol.

Table 1 is representative of the results obtained. In this experiment the temperature of medication was 20 C. A filtered 24-hour extract-

[9] Bull. Manila Med. Soc., 1911.
[10] Personal communication.
[11] Med. Rec., 1915, 87, p. 476.
[12] Jour. Infect. Dis., 1911, 8, p. 1.

broth culture of B. typhosus was used, the proportion of culture to disinfectant being 0.1 c.c. to 5 c.c. Extract broth was employed as subculture medium, reaction neutral to phenolphthalein. Results were read after 24 hours.

TABLE 1

Results of an Experiment Showing Bactericidal Action of Emetin Against B. Typhosus with Rideal-Walker and Hygienic Laboratory Methods

Sample	Dilution	Results Over a Period of 15 Minutes					
		2½	5	7½	10	12½	15
Phenol......................	1:20	—	—	—	—	—	—
Phenol......................	1:30	—	—	—	—	—	—
Phenol......................	1:40	—	—	—	—	—	—
Phenol......................	1:50	+	+	—	—	—	—
Phenol......................	1:80	+	+	+	+	—	—
Phenol......................	1:100	+	+	+	+	+	+
Emetin....................	1:20	+	+	+	+	+	+
Emetin....................	1:30	+	+	+	+	+	+
Emetin....................	1:40	+	+	+	+	+	+
Emetin....................	1:50	+	+	+	+	+	+
Emetin....................	1:60	+	+	+	+	+	+
Emetin....................	1:100	+	+	+	+	+	+

Both methods yielded similar results. It is to be noted that a 1 : 20 solution of emetin failed to kill B. typhosus in the longest interval of exposure—15 minutes; whereas a 1 : 80 dilution of phenol proved germicidal in this time. In this respect our results confirm those of others, but further experiments have shown that emetin possesses antiseptic and germicidal properties when the time of exposure is longer continued. Nevertheless, it should be added here that Barrett and Campbell (personal statement) have found that B. typhosus was not killed by exposure to as low dilution as 1 : 200 of emetin hydrochlorid in neutral broth in 96 hours. The only difference apprehended was that these investigators did not use a filtered culture for inoculation and employed a different strain from that used in our experiments. Possibly clumping may have prevented proper penetration of the culture.

With the Test-Tube Method.—Further experiments were conducted with the test-tube method as used by Schamberg and Kolmer[13] for testing the germicidal activity of substances insoluble in water.

Solutions of emetin—4, 2, 1, 0.5, and 0.25%—were prepared in plain, sterile, neutral broth, and similar dilutions of pure phenol in distilled water. With a sterile 1-c.c. volumetric pipet definite amounts of a given dilution of germicide were placed in a series of 6 test tubes (plugged with cotton and sterilized beforehand) as follows: 0.1, 0.2, 0.4, 0.6, 0.8, and 1 c.c. Sterile broth was then added to each tube with a sterile 5- or 10-c.c. volumetric pipet until the total

[13] Jour. Am. Med. Assn., 1914, 62, p. 1950.

quantity in each tube was brought up to 4.9 or 9.9 c.c. To each tube and a control tube of plain broth without germicide, was added 0.1 c.c. of a 24-hour filtered culture of the test micro-organism. The tubes then contained 5 or 10 c.c., according to the dilution desired; usually we worked with 5 c.c.

Tables 2 and 3 show the dilutions of germicide, secured in this manner, that act on the bacteria added to the tubes.

TABLE   2

Dilutions of Germicidal Substance Obtained When the Total Amount is Made 5 c.c.

Stock Dilutions	Final Dilution When Diluent is Added to 5 c.c.					
	0.1 c.c.	0.2 c.c.	0.4 c.c.	0.6 c.c.	0.8 c.c.	1.0 c.c.
4%	1:1250	1:625	1:312	1:208	1:156	1:125
3%	1:1666	1:833	1:416	1:277	1:208	1:166
2%	1:2500	1:1250	1:625	1:416	1:312	1:250
1%	1:5000	1:2500	1:1250	1:833	1:625	1:500
0.5%	1:10,000	1:5000	1:2500	1:1666	1:1250	1:1000
0.25%	1:20,000	1:10,000	1:5000	1:3333	1:3500	1:2000

TABLE   3

Dilutions of Germicidal Substance Obtained When the Total Amount is Made 10 c.c.

Stock Dilutions	Final Dilution When Diluent is Added to 10 c.c.					
	0.1 c.c.	0.2 c.c.	0.4 c.c.	0.6 c.c.	0.8 c.c.	1.0 c.c.
4%	1:2500	1:1250	1:624	1:416	1:312	1:250
3%	1:3333	1:1666	1:833	1:277	1:416	1:333
2%	1:5000	1:2500	1:1250	1:832	1:624	1:500
1%	1:10,000	1:5000	1:2500	1:1666	1:1250	1:1000
0.5%	1:20,000	1:10,000	1:5000	1:3332	1:2500	1:2000
0.25%	1:40,000	1:20,000	1:10,000	1:6666	1:5000	1:4000

Higher dilutions than these may be obtained by using higher stock dilutions; finer gradations may be secured by using 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 c.c. of stock dilution.

The tubes were then incubated at 37 C., and the results read and recorded each day over a period of 8 to 10 days. The controls were first inspected; they showed a good growth after the first 24 hours. As a rule, it was sufficient to inspect the tubes to learn the results: when the broth was perfectly clear, the result was set down for that day as negative or "germicidal"; if cloudy, the result was positive, the dilution of substance having failed to kill the test micro-organism.

By cultivating the tubes over a period of 10 days it was possible to determine the germicidal values of the substance tested. During the first 4 or 5 days the bacteria grew up in successive dilutions, thereby showing that their multiplication had been hindered through an antiseptic action of the substance but not killed. Very occasionally the bacteria in a tube grew up after the 7th day, but in no instance did we observe this occurring after the 8th or the 10th day. After this time all clear tubes were subcultured on slants of plain or glucose neutral agar to test their sterility; at the same time a number of those in which bacteria had grown up were subcultured to determine whether or not the micro-organisms had undergone spontaneous death. These subcultures were made by transferring several 4-mm. loopfuls of broth to the

slant of agar. With the micro-organisms used by us the broth cultures (i. e. the controls) were found viable at the end of 10 days; in all instances tubes that had been clear on the 8th or the 10th day, proved sterile. In each experiment the controls and a number of the tubes in which bacteria had grown up were examined by means of stained smears and subcultures to determine the purity of the growth.

With this method different experiments conducted at different times and with the same stock culture of micro-organism yielded very similar results when similar dilutions were used. In other words, if 0.1 c.c. of a 2% solution in 5 c.c. (= 1 : 2500) proved germicidal for a certain micro-organism, this dilution was found uniformly germicidal through several experiments. But different dilutions compared with each other not infrequently showed discrepant results. For example, 0.1 c.c. of a 4% solution in 5 c.c. gave a dilution of 1 : 2500; likewise, 0.4 c.c. of a 0.5% solution in 5 c.c. gave a dilution of 1 : 2500. Yet a micro-organism might be killed in the test tube containing the first-mentioned dilution, and might not be killed in the second tube. We have reason to believe that these discrepancies were at times due to errors in technic, as with closer attention to details more uniform results were observed.

For purposes of illustration, the results of a single experiment, selected out of a large number that were conducted by the test-tube method, are shown in Table 4. In this experiment, emetin hydrochlorid and phenol were used, in stock solution of 1%, and a filtered 24-hour extract-broth culture of B. typhosus in dose of 0.1 c.c. to each tube. The culture medium and diluent were extract broth, reaction neutral. The quantity in each tube was sufficient to make a total of 5 c.c.

TABLE 4

Results of Experiment Showing Bactericidal Action of Emetin Hydrochlorid and Phenol Against B. Typhosus with the Test-tube Method

Solution	Dose,	Final Dilution	Results Over Period of 10 Days									
			1	2	3	4	5	6	7	8	9	10
Emetin hydrochlorid 1%	0.1	1:5000	—	+	+	+	+	+	⌐	+	+	+
	0.2	1:2500	—	+	+	+	+	+	+	+	+	+
	0.4	1:1250	—	—	+	+	+	+	+	+	+	+
	0.6	1:833	—	—	—	—	—	+	+	+	+	+
	0.8	1:625	—	—	—	—	—	—	—	—	—	—
	1.0	1:500	—	—	—	—	—	—	—	—	—	—
Phenol 1%	0.1	1:5000	+	+	+	+	+	+	+	+	+	+
	0.2	1:2500	+	+	+	+	+	+	+	+	+	+
	0.4	1:1250	+	+	+	+	+	+	+	+	+	+
	0.6	1:833	—	+	+	+	+	+	+	+	+	+
	0.8	1:625	—	—	—	—	—	—	—	—	—	—
	1.0	1:500	—	—	—	—	—	—	—	—	—	—

In this experiment emetin proved antiseptic in a dilution of 1 : 5000, all tubes remaining perfectly clear after the first 24 hours' incubation, whereas the control tube showed a good growth; at the end of the

10-day period of observation the dilution of 1 : 625 was sterile. Similar results with emetin and B. typhosus were observed in other experiments.

As noted in the table, phenol also proved germicidal for B. typhosus in dilution of 1 : 625. Phenol proved germicidal quickly if at all; that is, tubes found sterile at the end of 48 hours were likely to remain so over the 10-day period of observation. With emetin, however, as before stated, the germicidal action was much slower, so that it was necessary to observe the results over the 10-day period in order to secure accurate determinations.

The results of experiments with emetin and phenol and the various test micro-organisms are summarized in Table 5. The dilutions given are those which proved germicidal through the 10-day period of observation. As previously stated, the results varied slightly with different stock solutions of the same substance, and for this reason the results observed with each solution are placed in the tables.

TABLE 5

Summary of the Results in the Study of the Germicidal Activity of Emetin Hydrochlorid and of Phenol

Substance Used	Test Micro-organisms	Stock Solutions				
		4%	2%	1%	0.5%	0.25%
Emetin hydrochlorid	B. typhosus......	1:624	1:625	1:625	None*	None†
	Staphylococcus aureus	1:2500	1:2500	1:5000 to 1:1250	1:2500 to none in -1:1000	None
	Streptococcus salivarius	1:624	1:2500	1:2500	1:2500	1:2500 to none
	B. anthracis.....	At least 1:125 to 1:1250	At least 1:2500	1:5000 to 1:1250	1:5000 to 1:6666	1:2000
	B. subtilis.......	At least 1:1250	At least 1:2500	1:5000 to 1:1250	1:2500	1:2000 to none
Phenol	B. typhosus.....	1:624	1:500	1:625	None	None
	Staphylococcus aureus	1:624	1:416 to none in 1:250	None in 1:500	None	None
	Streptococcus salivarius	1:312	1:416	1:500	None	None
	B. anthracis.....	1:312 to 1:625	1:416	1:500	None	None
	B. subtilis.......	1:312	1:416	1:500	None	None

* No germicidal action in the lowest dilution, 1 : 1000.
† No germicidal action in the lowest dilution, 1 : 2000.

From a study of Table 5 it is evident that with the technic employed, whereby the substance is left in contact with the test micro-organism over the entire period of observation, emetin proved equal to phenol in germicidal power, and was frequently from 1 to 5 times more effi-

cacious than phenol in this respect. The striking results observed with the spore-bearing B. anthracis and B. subtilis suggest that while emetin is a slowly acting germicide, it is capable of attacking and killing the naked germs in spore-germination.

In other experiments 2, 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, to $\frac{1}{512}$% solutions of emetin, in amounts of 2 c.c. each, in a series of sterile test tubes, prepared with sterile neutral broth as the solvent and diluent, were inoculated with 0.1 c.c. of a filtered 24-hour broth culture of B. typhosus; at the end of 10 days the tubes containing 2, 1, $\frac{1}{2}$, and $\frac{1}{4}$% solutions were sterile; all others, including the control, showed a good growth of the bacillus. After the first 24 hours all dilutions from 2 to $\frac{1}{16}$% inclusive were sterile, but during the succeeding days the $\frac{1}{16}$ and $\frac{1}{8}$% solutions permitted the bacillus to grow. In a similar experiment conducted with phenol, $\frac{1}{8}$% was the highest dilution proving germicidal, and was therein superior to emetin, the highest germicidal dilution of which was $\frac{1}{4}$%.

With the Plate Method.—We have studied the germicidal activity of emetin and of phenol (as control) in plates of agar agar, after the following method: Stock solutions of emetin varying from 4 to $\frac{1}{4}$% were prepared in sterile broth as heretofore. A given dilution being used, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 c.c. were placed in a series of sterile Petri dishes. To each dish and the control was added 0.01 c.c. of a filtered 24-hour extract-broth culture of the test microorganism. A flask of plain neutral agar was melted and cooled to 42 C., and by means of a sterile 10-c.c. volumetric pipet sufficient of this agar was added to each plate to make the total quantity in each, 10 c.c., or as close to this amount as was possible when pipetting agar at this temperature. Each plate was thoroughly mixed and all incubated at 37 C.; counts were made at the end of 24, 48, and 72 hours.

Table 6 shows the results of a single experiment conducted in this manner. Emetin hydrochlorid and phenol were used, in stock dilution of 2%, and a filtered 24-hour extract-broth culture of B. typhosus in dose of 0.01 c.c. to each plate. In the experiment with phenol plain agar agar (1.5%) was used, reaction neutral. The quantity in each plate was sufficient to make a total of 10 c.c.

The great advantage of the plate method over the test-tube method consists in the worker's ability to count colonies in the plates, and thus to detect finer degrees of germicidal activity. In the test-tube method the result is either positive or negative without any intervening ground.

TABLE 6

Results of Experiment Showing Bactericidal Effect of Emetin Hydrochlorid and of Phenol on B. Typhosus with the Plate Method

Solutions	Dose	Final Dilution	Results of Plate Counting Over a Period of 72 Hours		
			24 Hr.	48 Hr.	72 Hr.
Emetin hydrochlorid 2%	0.1	1:5000	Uncountable*........	Uncountable.........	Uncountable
	0.2	1:2500	Uncountable.........	Uncountable.........	Uncountable
	0.4	1:1250	32,000................	Uncountable.........	Uncountable
	0.6	1:832	Sterile................	Uncountable.........	Uncountable
	0.8	1:624	Sterile................	Sterile................	Sterile
	1.0	1:500	Sterile................	Sterile................	Sterile
Phenol 2%	0.1	1:5000	Uncountable.........	Uncountable.........	Uncountable
	0.2	1:2500	Uncountable.........	Uncountable.........	Uncountable
	0.4	1:1250	2800................	5000................	11000
	0.6	1:832	Sterile................	Sterile................	Sterile
	0.8	1:624	Sterile................	Sterile................	Sterile
	1.0	1:500	Sterile................	Sterile................	Sterile

* Too many colonies to make a count reliable.
The controls were "uncountable" after the first 24 hours' incubation.
As in the test-tube method, finer gradations in germicidal activity may be secured by using 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 c.c. of the stock solution.

In Table 7 are given the results observed with emetin and with phenol with B. typhosus and Staphylococcus aureus after 72 hours with this technic.

TABLE 7

Results of Experiment Showing Bactericidal Activity of Emetin and of Phenol Against B. Typhosus and S. Aureus with the Plate Method

Micro-organism	Solutions	Results After 72 Hours	
		Emetin	Phenol
Bacillus typhosus..............	4%	1:312*	1:624*
	2%	1:624*	1:832*
	1%	Uncountable (1:1000)†	3000 colonies (1:1000)
	0.5%	Uncountable (1:2000)	Uncountable (1:2000)
	0.25%	Uncountable (1:4000)	Uncountable (1:4000)
Staphylococcus aureus.........	4%	Uncountable (1:250)†	1:624*
	2%	Uncountable (1:500)	1:500*
	1%	Uncountable (1:1000)	Uncountable (1:1000)
	0.5%	Uncountable (1:2000)	Uncountable (1:2000)
	0.25%	Uncountable (1:4000)	Uncountable (1:4000)

* Plates sterile in these dilutions.
† The lowest dilution put up with this stock dilution; so many colonies present that an accurate count could not be made.

If Table 7 is compared with Table 5, it will be noted that emetin proved less germicidal with the plate method (or in a solid medium) than with the test-tube method (or in a fluid medium); especially was this true with the culture of S. aureus. It is probable that the drug diffuses slowly, so that in solid media the test micro-organisms escape destruction and multiply to a greater extent than in fluid media. Dr. Robert A. Keilty (personal communication), testing out emetin against

B. coli and an undetermined diplococcus by the method of Cheyne[14] (which is partly a test of diffusibility of germicidal substances) and a 1 : 200 incorporation of emetin, found emetin practically without germicidal power, these results corresponding to our own as shown in Table 7.

Germicidal Action of Emetin on the Micro-organisms in Pyorrheal Pus.—As the amount of organic matter, particularly pus, in the diluent and menstruum of a test for germicidal activity modifies the results—through absorption of the drug, formation of new and inert compounds, etc.—we have tested the germicidal activity of emetin against the micro-organisms found in pus, using a salt-solution suspension of pus from the mouths of persons suffering with severe forms of pyorrhea alveolaris.

Ten cubic centimeters of normal salt solution were placed in a test tube, warmed to 37 C. and kept at this temperature. This was then inoculated with pus—secured from pus pockets by means of a heavy, sterilized platinum-wire spade—until the suspension was decidedly milky. This suspension was shaken with sterile beads for 10 minutes and used without filtration. Smears showed a variety of micro-organisms, including gram-positive cocci and diplococci, gram-negative diplococci, gram-positive and negative bacilli, and numerous spirochetes and amebae. The emulsion was ready for use within 15 minutes after securing of the pus, was kept constantly at or about 37 C., and in density corresponded approximately to a bacterial vaccine containing 5 billion bacteria to the cubic centimeter.

TABLE 8

Results of Experiment Showing Bactericidal Activity of Emetin and of Phenol Against Micro-organisms in Pus from Pyorrhea Alveolaris

Solutions	Dilution	Results Over a Period of 90 Minutes						Phenol Coefficient
		1	15	30	45	60	90	
Emetin......	1:50	+	+	+	−	−	−	200:400 = coefficient 2
	1:100	+	+	+	+	+	−	
	1:200	+	+	+	+	+	−	
	1:400	+	+	+	+	+	+	
	1:800	+	+	+	+	+	+	
Phenol.......	1:50	−	−	−	−	−	−	
	1:100	+	−	−	−	−	−	
	1:200	+	+	+	−	−	−	
	1:400	+	+	+	+	+	−	
	1:800	+	+	+	+	+	+	

All controls showed heavy growths in 24 hours.

Five dilutions (4, 2, 1, 0.5, and 0.25% solutions) of emetin were prepared in normal salt solution, and 1 c.c. of each placed in each of 5 sterile test tubes. To each tube was added 1 c.c. of the emulsion of pus and bacteria; the whole was then shaken gently, and kept in a water bath at a constant temperature of 35 to 37 C. The final dilutions of emetin acting on the bacteria were then 2% (1:50); 1% (1:100); ½% (1:200); ¼% (1:400); and ⅛% (1:800) solutions. At the end of 5, 15, 30, 45, 60, and 90 minutes each tube was subcultured into a tube containing 10 c.c. glucose broth by transfering a 4-mm.

[14] Brit. Med. Jour., 1912, 1, p. 1424. Lancet, 1912, 2, p. 1062. Therap. Gaz., 1912, 36, p. 837.

loopful. Controls of the pus and bacterial emulsion were prepared at the beginning and after the completion of the experiments, by subculturing the emulsion in the same manner. A like experiment was conducted with phenol, the same pus and bacterial emulsion being used.

The results as read at the end of 48 hours are shown in Table 8.

With the 90-minute interval of exposure phenol proved twice as strongly germicidal as emetin. It is worthy of particular note that even a 2% solution of emetin required an exposure of 45 minutes to effect sterilization of the bacterial emulsion, whereas phenol killed in 5 minutes in this dilution, and in 15 minutes in a 1% dilution. The 0.5% solution of emetin, which is commonly used in the treatment of pyorrhea alveolaris, required an exposure of 1.5 hours in this experiment (at a temperature of 35 to 47 C.) to effect sterilization of the bacteria in pyorrheal pus. If these experiments may be accepted as a criterion of the germicidal activity of emetin in the diseased tissues, it is apparent that this activity, while present, is relatively slight, and requires that the emetin remain in contact with the tissues over a considerable period of time.

AMEBACIDAL ACTIVITY OF EMETIN HYDROCHLORID WITH REFERENCE TO ENDAMOEBA GINGIVALIS, GROS

The generally accepted specific amebacidal influence of emetin, which had as its experimental basis the studies of Vedder[6] on the influence of ipecac and emetin on amebae in culture, was substantiated later by experiments of Wherry[7] and others, and brilliantly demonstrated by Sir Leonard Rogers[14] and subsequently by a great number of clinicians in application to cases of amebic dysentery. Its value in the treatment of amebic pyorrhea has been shown by Smith and Barrett,[8] Bass and Johns,[2] and others. The impossibility to date of obtaining pathogenic amebae in successful culture has prevented precise experimentation in this as in other lines of interest in relation to these protozoa; and the information we possess concerning the effects of emetin on both Endamoeba histolytica, Schaudinn, and Endamoeba gingivalis, Gros, is derived from clinical observation rather than from laboratory experimentation. If we were permitted to judge purely from clinical results, and to hold as basis for calculation that in an average adult human being there are, at the least, twenty pounds of fluid capable of acting as solvent and diluent of hypodermatically introduced emetin hydrochlorid, and that successful amebacidal influence has at times followed

as low a daily dosage as one-fourth grain of the drug both in dysentery and in pyorrhea (remembering, too, that it is unlikely that all of an injected dose will at once be diffused from the site of introduction, and making allowance for a reasonable but unknown rate of elimination and fixation), it would be well within reason to believe that such occasional successes are attained by dilutions of the remedy ranging well above 1 : 500,000. But in a precise way we know practically nothing of the quantitative relations of the remedy to these parasites, of the time requirements, or even of the exact mode of attack. James[15] has pointed out, from clinico-pathologic studies on the intestinal amebae, that in the course of the administration of emetin cytoplasmic and nuclear degenerative changes are manifested by the amebae obtained from the dejecta; and one of us,[8] in watching the unstained oral amebae in pyorrheal pus under the microscope, observed that when an emetin solution was allowed to flow under the cover into contact the amebae rapidly became quiescent and rounded, their substance assuming a hyaline and relatively opaque appearance, the clear ectoplasm first manifesting the change, which apparently increased at the expense of the endoplasm, until practically the entire parasite, except possibly a few of its contained globules, which became condensed in the central part of the cell, had become opaque and glossy.

This change was at first believed to indicate the actual death of the parasite, and at present the writers believe that when it is well developed it usually does mean that the protozoan is dead; but we have had ocular proof that some of the amebae which show this change, in at least a mediate but readily recognizable degree, may still retain the power of movement and are therefore still living. If it were possible to grow these organisms artificially and after definite exposure to make subcultures of the exposed material in order to determine the destruction of the amebae or their persistent viability, matters would be very different; in the lack of such ability we believed it worth while to proceed on the assumption that such changes usually indicate death, or presage death, and that at least they show an influence by the drug on the parasite.

We therefore prepared solutions of emetin hydrochlorid in proportions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, and 1/2048% strength (1 : 200, 1 : 400, etc., up to 1 : 204800) in normal salt solution, and by means of pipets mixed as accurately and as thoroughly as possible equal parts of these with fresh ameba-bearing pus from pyorrheal pockets (all from same patient)

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[15] Amer. Jour. Tropical Dis., 1913, 1, p. 431.

on slides, covered them and sealed the cover with wax, and maintained them at 30 C. in an incubator during the period of observation. This mixture of pus and emetin solution rendered the available strength of the remedy in the preparations 1 : 400, 1 : 800, 1 : 1600, etc., to 1 : 409600. Control preparations made with normal salt solution replacing the emetin-salt solution were similarly made and cared for. In these, movement was apparent, of a sluggish type, as much as 8 hours after the acquirement of the material from the mouth, and the hyaline change in the parasites was not appreciable. Six hours, therefore, was regarded as a safe observation period.

The series reported in Table 9 will serve to indicate the general result.

TABLE 9

Influence of Different Dilutions of Emetin on Endamoeba Gingivalis, Gros, at 2-hour Intervals

Dosage Rates	Appearance of the Amebae Over a Period of 6 Hours		
	2 Hr.	4 Hr.	6 Hr.
1:400...............	Motionless Hyaline Rounded	Motionless Hyaline Rounded	Motionless Hyaline Rounded
1:800.........................	Motile Hyaline Pseudopodia	Motionless Hyaline Rounded	Motionless Hyaline Rounded
1:1600...............	Motionless Hyaline Pseudopodia	Motionless Hyaline Rounded	Motionless Hyaline Rounded
1:3200.........................	Motile Hyaline Pseudopodia	Motile Hyaline Pseudopodia	Motile Hyaline Pseudopodia
1:6400.......	Motile Hyaline (clear cytoplasm) Pseudopodia	Motile Hyaline Pseudopodia	Motile Hyaline Pseudopodia
1:12800.........................	Motionless Hyaline Pseudopodia	Motionless Hyaline Pseudopodia	Motionless Hyaline Rounded
1:25600........	Motile Hyaline Pseudopodia	Motionless Hyaline Pseudopodia	Motionless Hyaline Rounded
1:51200.........................	Motile Clear cytoplasm (hyaline) Pseudopodia	Motionless Hyaline Rounded	Motionless Hyaline Rounded
1:102400.........................	Motile Clear cytoplasm (hyaline) Pseudopodia	Motionless Hyaline Rounded	Motionless Hyaline Rounded
1:204800.........................	Motile Hyaline (clear cytoplasm) Pseudopodia	Motionless Hyaline Rounded	Motionless Hyaline Rounded
1:409600.........................	Motile Hyaline (clear cytoplasm) Pseudopodia	Motionless Hyaline Rounded	Motionless Hyaline Rounded

The discovery of any motile amebae in a slide was noted as if all were motile, altho this really was far from true, as some motionless, hyaline, round examples were found in all specimens. Throughout the entire observation period active motility of certain flagellates (tricho-monads mainly) persisted even in the lower dilutions. Movement of associated motile bacteria was lost practically without reference to the emetin proportions before the close of observation. The persistence of motility in dilutions of 1:3200 and 1:6400 is believed to have been due to a failure properly to mix the pus and emetin solution, as both were rather thick samples, and the motility was particularly noted in amebae well embedded in the denser fields. We are unwilling to say at what time and at what dosage death of the amebae takes place; we are willing to assert that at even the highest dilution employed emetin attacks the amebae and brings about visible structural change within 2 hours (for the lower dilutions we know that this may take place within a few minutes); we believe that these changes are at least prelethal. If we are correct in this belief, our results tally fairly with the rough quantitative conclusions which, as we have suggested, may be drawn from clinical sources.

TRYPANOCIDAL ACTIVITY OF EMETIN HYDROCHLORID

While the highly specific influence of emetin has been generally recognized and is sustained by the observations described, it was con-sidered worth while to include here a brief study of the trypanocidal activity of the drug, not only as a matter of interest in its effect on trypanosomes themselves, but as a means of adding in some measure to our knowledge of its general protozoacidal influence. It was thought, too, that, in view of the suspected relation between these flagellates and spirochetes and the known coincidence of chemotherapy, as exem-plified by salvarsan, for treponemata, spirochetes, and in some measure for these higher flagellates, some suggestions might be available from such a study as to the possible influence of emetin on the spirochetes of the mouth. This, of course, is far from being of direct value; and since by the method of Noguchi it is possible to obtain cultures of the various mouth spirochetes, we hope later to be in position to present direct, available data on this relation. Experiments of a preliminary charac-ter, conducted along lines similar to those in the following trypanosome study, with thick emulsions of pyorrheal pus showing numerous and various spirochetes as well as bacteria, have yielded suggestive results.

The motility of the spirochetes could easily be seen; and, in general, dilutions of emetin of from 1 : 400 to 1 : 12,000 seemed to show spirochetacidal influence within an hour, as far as could be judged on the basis of motility alone.

For the study of trypanocidal influence T. lewisi and T. equiperdum were selected and the following technic worked out for conducting these experiments in vitro.

White rats were infected with the respective strains, and used when a drop of blood from the tail showed, on microscopic examination, large numbers of the trypanosomes. Blood was then secured from the tail or from the heart of the animal, and enough placed in a tube of 1% sodium citrate in normal salt solution warmed to 40 C. for each loopful of emulsion, examined in hanging drop with a 1/6 objective and No. 4 eyepiece (Leitz), to show at least 10 trypanosomes in each field. The blood-trypanosome emulsion was kept at a constant temperature of 40 C. by standing the test tube in a beaker of water at this temperature.

Dilutions of emetin varying from 2% (or 1 : 50) to approximately $\frac{1}{500}$% (or 1 : 51200) were prepared in warm normal salt solution. One loopful of each dilution of emetin was mixed on a warm cover slide with an equal loopful of trypanosome emulsion, and a hanging drop preparation made and sealed with vaselin, as in conducting the microscopic agglutination test with typhoid bacilli. The final dilutions of emetin acting on the trypanosomes then ran from 1% (1 : 100) to 1/1024% (1 : 102,400). Each slide and the controls were marked with the time at which the emetin and trypanosomes were mixed. The slides were placed in an incubator at 40 C. and examined on a warm stage with 1/6 objective and No. 4 eyepiece (Leitz).

In all preparations at least 5 trypanosomes could be seen in each field. In the controls the parasites were always vigorous and actively motile; after 2½ hours, however, the motility was much decreased and for this reason each experiment was terminated within this time.

At varying intervals the slides were examined and the effect of the emetin on the trypanosomes studied. The results were definite and easily read within the limits and defects of this technic. First the trypanosomes lost their active to and fro movements, and remained in one position with constant vibratile movement. Later the latter movement became more and more sluggish and finally ceased. The bodies of the parasites at first were elongated, but later became short and swollen, so that various bizarre and peculiar forms could be seen. As soon as careful examination of a slide showed total loss of motility in all trypanosomes, the time was noted; and for the purpose of these experiments the trypanosomes were regarded as having been influenced by the emetin. We realize the defects of this method of study, chiefly in that absolute loss of motility is no sure indication that the trypanosomes are dead and that disintegration will occur. (We have experi-

mented in a different manner by mixing definite volumes of trypano-
some suspensions in sterile test tubes with various dilutions of
emetin and after a definite interval of time removing the trypanosomes
by centrifugation, washing once with normal salt solution to remove
traces of emetin, and injecting the trypanosomes re-suspended in
warm salt solution into the peritoneal cavities of a series of rats.
The results, however, were irregular, a number of controls remaining
sterile; the necessarily large amount of handling and the resultant cool-
ing or chilling of the emulsion in centrifugation had killed the
trypanosomes.)

The results of these experiments are summarized in Table 10. The
time required to bring about total loss of motility varied in different
experiments with trypanosomes of the same strain but from different
seed rats. The intervals shown in the table represent the shortest and
longest intervals of time, respectively, to bring about these results with
the various dilutions of emetin.

TABLE   10
Trypanocidal Activity of Emetin

Dilutions	Minutes of Time Required to Kill All Trypanosomes	
	T. lewisi	T. equiperdum
1:100	3 to 10	17 to 55
1:200	10	13 to 32
1:400	12	11 to 19
1:800	14	21 to 30
1:1600	15 to 18	30 to 41
1:3200	20 to 40	40 to 50
1:6400	25 to 45	60 to 72
1:12800	30 to 70	100 to 130
1:25600	60 to 90	125 to 140
1:51200	70 to 120	150 to 190
1:102400	80 to 140	

These experiments show that emetin possesses trypanocidal
activity. With one hour as a safe interval of exposure, since in that
space the controls showed no appreciable spontaneous deterioration, a
dilution of emetin of 1 : 25,000 was destructive for T. lewisi and a
dilution of 1 : 6000 for T. equiperdum. The higher resistance of the
pathogenic T. equiperdum is probably to be ascribed to a state of higher
vitality and vigor in this parasite. In both cases, however, the
trypanocidal activity of emetin in vitro appears to be lower than its
amebacidal activity and this was even more evident in our experiments
in vivo, which are reported in a separate communication.

DISCUSSION

Interest in this study pertains to the rôle of emetin hydrochlorid not only as an amebacide, but likewise as a bactericide in the treatment of pyorrhea alveolaris and amebic dysentery. We were surprised at the high grade of bactericidal action possessed by emetin, altho this activity is not apparent unless the drug remains in contact with bacteria, preferably in a fluid medium, over relatively long intervals of time.

In the treatment of pyorrhea alveolaris by local application of emetin, the drug should not be used in solutions stronger than 0.5% on account of its local irritant effects on the tissues. Tho 0.25% is germicidal in the test tube, this action is apparent only when the solution of emetin is left in contact with the test micro-organism; a 5% solution, on the other hand, fails to kill B. typhosus in 15 minutes. In view of the fact that most of the drug must be ejected from the pus pockets in the gums, by reason of the movements of the jaw, within a short time after the application has been made, it is reasonable to suppose that the quantity of emetin remaining for a sufficient length of time to exert a bactericidal action must be small indeed.

On the other hand, emetin possesses a very high amebacidal action, as determined not only by studies in vitro, but likewise by studies of the material from pyorrhea alveolaris and from amebic dysentery following the hypodermatic injection of the drug. With a dose of 0.016 gm. administered in this manner to a 60 kilo man, the dilution in the body must be at least 1 : 500,000 or 1 : 1,000,000, if one considers that the total blood and body fluids may be placed conservatively at 20 pounds, and does not allow for local fixation of part of the drug at the site of injection—believing that a constant elimination proceeds. In view of the undoubtedly beneficial results following the hypodermatic injection of the drug and its demonstrated amebacidal powers (with which tentatively we relate the structural influence we have noted), it is reasonable to conclude that the drug exerts its curative effects largely by reason of its amebacidal properties, as in these dilutions a bactericidal action in vitro could not be demonstrated and our studies on the bactericidal action of emetin in vivo were largely negative in their results.

Briefly then, we are of the opinion that emetin hydrochlorid exerts some bactericidal action when applied locally in the treatment of pyorrhea alveolaris, but that its bactericidal activity is entirely secondary to its amebacidal action, being of probably even less influence

when emetin is administered hypodermatically. As far as this may, it means, moreover, that this large margin of specific influence of eme- tin on amebae is confirmatory of the belief that these oral amebic para- sites are an important factor in the pathogenesis of pyorrhea. It does not prove that they are the sole agents by any means ; and for other reasons the writers are disposed to maintain the attitude expressed by Smith and Barrett, quoted at the opening of this paper, as to a probable association of etiologic factors.

A logical conclusion would seem to be that pyorrhea alveolaris should be treated locally with emetin, with or without coincident hypodermatic injections of the drug ; clinical observations bear out the correctness of this conclusion, as in our experience best results are secured with the local use of the drug combined, in cases accompanied by com- plications, with hypodermatic medication.

CONCLUSIONS

Emetin hydrochlorid possesses bactericidal properties, but prolonged contact with bacteria is required before this action becomes apparent. A 5% solution of emetin failed to kill B. typhosus in 15 minutes, but with a special technic, in which the drug remains in contact with the test micro-organisms, emetin proved about equal to, or even on occasion 5 times more antiseptic and germicidal than corresponding dilutions of pure phenol.   .

The bactericidal activity of emetin is more apparent in fluid than it is in solid culture media.

In an emulsion of pus and various bacteria from pyorrhea alveolaris a 2% solution of emetin required 45 minutes to effect sterilization, whereas a corresponding dilution of phenol proved germicidal in 5 minutes or less ; a 0.5% solution of emetin required $1\frac{1}{2}$ hours, and a corresponding dilution of phenol, 45 minutes, to sterilize the emulsion.

Emetin hydrochlorid possesses trypanocidal properties in vitro, but this action is probably less vigorous than is its amebacidal action.

Emetin is highly amebacidal, producing a marked structural change in Endameba gingivalis when applied in direct contact, even in high dilution.

Emetin hydrochlorid probably exerts some bactericidal action when applied locally in the treatment of pyorrhea alveolaris ; but its bacteri- cidal activity must be entirely secondary in importance to its ameba- cidal action, in view especially of the beneficial results and the dis-

appearance of amebae following the hypodermatic use of the drug in the treatment of pyorrhea alveolaris and amebic dysentery when the drug is highly diluted in the body fluids.

In view, however, of the probable bactericidal value of emetin when applied locally it would appear that the logical treatment of pyorrhea alveolaris should consist primarily in its local application combined with hypodermatic administration, especially in severe infections or in those accompanied by systemic complications.

THE BACTERICIDAL AND PROTOZOACIDAL ACTIVITY OF EMETIN HYDROCHLORID IN VIVO *

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Having found that emetin hydrochlorid possesses some bactericidal and trypanocidal power in vitro, as shown in our previous communication,[1] we have investigated the possibility of its exerting similar influence in vivo.

Emetin has been used successfully in the treatment of amebic dysentery by hypodermatic injection from the time of its introduction by Rogers,[2] and the studies of Vedder[5] and others leave no doubt regarding the high specificity and the parasitropic action of ipecac and emetin for amebae. Rogers, it is said, was preceded in the use of emetin in dysentery by Dr. W. S. Eccles in India in 1869,[3] and Harris is said also to have employed the remedy at an early date,[4] at Simla. However, the work of Rogers, independent of these and really based on Vedder's studies, is actually the basis for the modern clinical use of emetin in amebic dysentery and its complications. Bass and Johns[6] have advocated the hypodermatic injection of emetin in the treatment of amebic pyorrhea and, while this form of treatment has not proved in our experience as successful as the local application of emetin, yet there is abundant evidence showing the amebacidal and curative action of this drug when so administered.

In addition to this amebacidal activity of emetin,[7] the improvement of various arthritic, neuritic, digestive, hemic, and other complications of pyorrhea alveolaris (attributed to a condition of toxemia) following the local application and especially the hypodermatic injection of emetin, as reported by Evans and Middleton; the amelioration of symptoms and some improvement in patients with pulmonary tuberculosis under treatment with emetin, as reported by Tatchell;[8] and the value of emetin administered subcutaneously as a form of abortive treatment of typhoid fever, as reported by Frazier[9]—all suggest that emetin may exert some bactericidal action in vivo.

The view generally held by those who have noted the beneficial influence of *hypodermatically* administered emetin on various complications of amebic dysentery and amebic pyorrhea has been to the effect that the drug was mainly amebacidal and that as a result of the destruction of amebae there was coincident cessation of the formation of amebic and bacterial toxins and

* Received for publication September 21, 1915.

[1] Jour. Infect. Dis., 1916, 18, p. 247.

[2] Brit. Med. Jour., 1912, 1, p. 1424; 2, p. 405.

[3] Practitioner, 1914, 93, p. 4.

[4] Indian Med. Gaz., 1914, 49, p. 193.

[5] Jour. Am. Med. Assn., 1914, 62, p. 501 (full bibliography to literature).

[6] Ibid., 1915, 64, p. 553.

[7] Ibid., p. 422.

[8] China Med. Jour., 1915, 29, p. 167.

[9] Med. Rec., 1915, 87, p. 476.

of the mechanical conveyance of bacteria into deeper tissues. Smith and Barrett[10] believe that the amebae act in symbiotic relation with some or all of the micro-organisms with which they are associated in nature, and that the amebae by their digestive processes not only furnish a highly fitting pabulum for the growth of these bacteria but aid in setting free bacterial toxins which cause local necrosis and may be productive of a widespread series of complications. The destruction of amebae would be followed therefore by diminished toxin-production, by the removal of a source of food supply for bacteria, and by the loss of a means of their mechanical conveyance into deeper tissues through the migratory habits of the amebae.

SCOPE OF THIS INVESTIGATION

We have studied the bactericidal action of emetin in vivo on Staphylococcus aureus, Bacillus tetanus, and Bacillus anthracis. These bacteria were selected because of the ease with which the lethal dose of each may be determined, the certainty of their pathogenicity for experimental animals, and the relatively high bactericidal action of emetin in vitro on spore-forming bacteria.

The trypanocidal activity of emetin in vivo was studied with Trypanosoma equiperdum and T. lewisi. The amebacidal action of emetin was of minor importance in this investigation by reason of the large amount of work that had been done in this direction and the unanimous weight of opinion regarding the highly specific amebacidal action of ipecac and emetin.

In studying the influence of emetin on Staphylococcus aureus in vivo, rabbits were used; for B. tetanus and B. anthracis, white mice were employed; and for T. equiperdum and T. lewisi, white rats.

The Lethal Dose of Emetin

In many of our experiments emetin was administered intravenously, and in all instances the doses were based on the weight of the animal (rabbits, mice, and rats), being so graded that each animal received a certain quantity per 60 kilos of body-weight (about 132 pounds, adopted as an average weight for adult human beings).

By intravenous injection the immediate lethal dose of emetin for white rats was found to be about 0.016 gm. (¼ grain) per kilo of body-weight, which is equivalent to 0.96 gm. (about 15 grains) per 60 kilos (132 pounds). In this animal a dose equivalent to 1.17 and 1.3 gm. (18 and 20 grains) per 60 kilos of body-weight was invariably fatal, while most animals withstood a dose equivalent to 0.77 gm. (12 grains) or 0.0129 gm. per kilo (equivalent to about ⅕ grain per kilo).

Emetin was found slightly more toxic for rabbits. A dose equivalent to from 0.77 to 0.96 gm. (12 to 15 grains) per 60 kilos of weight (.013 gm. or ⅕ to .016 gm. or ¼ grain per kilo) was usually immediately fatal on intra-

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[10] Oral Health, 1915, 5, p. 137.

venous injection; one rabbit succumbed to a dose equivalent to 0.51 gm. (8 grains) per 60 kilo of weight (equivalent to about .009 gm. or $\frac{1}{7}$ grain per kilo). In our experiments the immediate lethal dose of emetin for rabbits was from 0.65 gm. to 0.78 gm. (10 to 12 grains) per 60 kilos, which are equivalent to 0.01 to 0.0129 gm., (about $\frac{1}{6}$ to $\frac{1}{5}$ grain) per kilo.

As emetin hydrochlorid is usually administered to man by subcutaneous injections in doses of from 0.016 gm. to 0.065 gm. ($\frac{1}{4}$ to 1 grain), it is relatively free of organotropic action in so far at least as immediately harmful effects are concerned.

BACTERICIDAL ACTION OF EMETIN HYDROCHLORID IN VIVO

On Staphylococcus aureus.—In our first experiments rabbits were given an intravenous injection of 1 c.c. of a 24-hour broth culture of virulent Staphylococcus aureus—filtered through sterile filter paper to remove large clumps of the cocci. Two hours later emetin, dissolved in sterile normal salt solution, was administered in varying doses by intravenous injection to all animals except the controls. Three to five days later the animals were chloroformed and examined macroscopically and microscopically for staphylococcus abscesses in the kidneys, heart, peritoneum, etc.

In Table 1 are shown the results of this experiment:

TABLE 1

The Action of Emetin on Staphylococcus aureus in Vivo

Rabbit	Weight (Grams)	Dose of Emetin* (Grams)	Equiv- alent in Grains per 60 K. (or 132 lb.) Body Weight	General Condi- tion	Died	Autopsy 3 Days Later
1	1317	.0559	4	Good	......	Abscesses in kidneys, heart, etc.
2	1580	.005	3	Good	......	Abscesses in kidneys, heart, etc.
3	1255	.00256	2	Good	......	A few microscopic abscesses in one kidney
4	1370	.00146	1	Good	......	A few microscopic abscesses in both kidneys
5	1178	Control	0	Good	3rd day	Abscesses in kidneys, heart, pleura, etc.
6	1258	Control	0	Poor	......	Abscesses in heart, kidneys, pleura, etc.

* Single dose 2 hours after infection.

They may be considered generally of a negative character. One noticeable feature was the evident toxemia of the controls as compared to the well-being of the animals receiving emetin. Whether or not the absence of macroscopic abscesses in the organs of Rabbits 2 and 4 may be attributed to some bactericidal action of emetin is difficult to state. Since microscopic abscesses were found, we are inclined to

ascribe the results to a higher resistance on the part of these animals to the cocci.

In order to test this conclusion by a more delicate experiment, a second series of rabbits was given emetin intravenously immediately before the injection of staphylococci, and the same dose repeated at daily intervals for 3 doses, also by intravenous injection. In this experiment the same culture of Staphylococcus aureus was used, but the infecting dose was made 0.5 c.c. of a filtered 24-hour broth culture, instead of 1 c.c. as used in the preceding experiment.

The results are shown in Table 2:

TABLE   2

The Action of Emetin on Staphylococcus aureus in Vivo (Multiple Doses of Emetin)

Rabbit	Weight (Grams)	Dose of Emetin (Grams)	Equiv-alent in Grains per 60 K. (or 132 lb.) Body Weight	Number of Doses	Total Amount of Emetin Admin-istered	General Condi-tion	Died	Autopsy 5 Days After Infection
1	1147	.0097	8	1	......	......	Immedi-ately	Lethal dose of emetin
2	1354	.0057	4	4	.0228	Slightly toxic	......	A few abscesses in kidneys
3	1084	.0023	2	4	.0092	Good	......	Abscesses in kidneys and heart
4	1211	.0013	1	4	.0052	Good	......	Abscesses in kidneys and heart
5	1000	Control	....	......	......	Fair	......	Abscesses in kidneys, heart, etc.
6	1034	Control	....	......	......	Fair	......	Abscesses in heart, kidneys, etc.

With these results after the daily administration of relatively large doses of emetin by intravenous injection, and with an infection as light as was consistent with constant effects, we must conclude that emetin possesses slight, if any, bactericidal effect in vivo on virulent staphylococci.

On Bacillus anthracis.—A culture of B. anthracis was cultivated in neutral broth for 24 hours, shaken briefly with sterile glass beads, and its lethal dose in 24 hours determined by injecting a series of white mice subcutaneously with amounts varying from 0.001 to 0.4 c.c. With this culture 0.05 c.c. proved fatal with regularity at the expiration of about 24 hours.

A series of mice was then infected with 0.1 c.c., or double the 24-hour lethal dose of anthrax culture, by subcutaneous injection; 2 hours later emetin was administered by intraperitoneal injection. The

results are shown in Table 3. They suggest that the deaths of Mice 1 and 2 were hastened by the large doses of emetin, that there was some slight bactericidal effect in Mice 3 and 4, and that the smaller doses exerted no influence (Mice 5, 6, and 7).

TABLE 3

Action of Emetin on B.ˑAnthracis

Mouse	Weight (Grams)	Dose* of Emetin (Grams)	Equivalent in Grains per 60 K. or 132 lb. Body Weight	Day of Death		
				1	2	3
1	25	.000267	10	+		
2	22	.000188	8	+		
3	21	.000128	6	—	+	
4	15	.000064	4	—	+	+
5	18	.000057	3	+		
6	19	.0000428	2	+		
7	18	.0000192	1	+		
8	20	Control	..	+		
9	25	Control	..	+		
10	22	Control	..	+		

* Single dose 2 hours after infection.

All these animals showed enormous numbers of anthrax bacilli in the capillaries of the liver, kidney, heart, etc.

This experiment was then repeated; a smaller infecting dose of a 24-hour broth culture of B. anthracis (0.05 c.c.) was subcutaneously injected, and emetin was administered intraperitoneally immediately before injecting the bacilli and again at daily intervals until the mice succumbed. After death autopsies were performed and the internal organs fixed, cut, and stained for anthrax bacilli. The results are shown in Table 4.

TABLE 4

Action of Emetin on B. anthracis (Multiple Doses of Emetin)

Mouse	Weight (Grams)	Dose of Emetin (Grams)	Equivalent in Grains per 60 K. or 132 lb. Body Weight	Total Amount of Emetin Administered	Number of Doses	Day of Death			
						1	2	3	4
1	25	.000321	12	.000641	2	—	+	+	
2	20	.000214	10	.000642	3	—	—	+	
3	18	.000153	18	.000659	3	—	—	+	
4	19	.000122	16	.000244	2	—	+		
5	21	.0000856	4	.000171	2	—	+		
6	18	.0000385	2	.000115	3	—	—	+	
7	17	.0000181·	1	.000072	4	—	—	—	+
8	22	Control	..	....	..	—	—	+	
9	24	Control	..	....	..	—	+		
10	18	Control	..	....	..	—	—	+	

According to these experiments we cannot ascribe to emetin any decided bactericidal value in vivo on the anthrax bacillus; altho it

would appear in this experiment and in that shown in Table 3 that when emetin is administered in very large doses slight antiseptic rather than true germicidal effects may be noted.

On Bacillus tetanus.—In working with the tetanus bacillus there was difficulty in infecting the animals with a lethal dose of the bacilli and spores which would be free as possible from preformed toxin. It was not reasonable to expect emetin to be antitoxic, and an excess of toxin might be expected to kill the animals without determining any possible influence of the drug on the bacilli and spores alone.

Neutralization of broth cultures of tetanus bacilli with antitoxin and separation of the bacilli by centrifugation did not yield satisfactory and constant results. After several preliminary trials with an anaerobic culture of the bacillus in a large tube of agar agar, we succeeded in determining the lethal dose by subcutaneous injection of an emulsion prepared with definite volume of the agar culture in 5 c.c. of sterile, neutral broth. Having determined the lethal dose for a 48-hour period, a series of white mice was infected by subcutaneous injection with double this dose, followed 1 hour later by increasing doses of emetin administered by intraperitoneal injection. The results of this experiment are shown in Table 5:

TABLE 5

Action of Emetin on B. tetanus

Mouse	Weight (Grams)	Dose* of Emetin (Grams)	Equivalent in Grains per 60 K. or 132 lb. Body Weight	Day of Death	
				1	2
1	20	.000171	8	—	—
2	22	.000141	6	—	-
3	24	.000128	5	—	+
4	25	.0000107	4	—	+
5	25	.0000802	3	—	+
6	22	.000047	2	—	+
7	19	.00002	1	—	+
8	22	Control	...	—	+
9	20	Control	...	—	+
10	18	Control	...	—	+

* Single dose 1 hour after infection.

This experiment was then repeated by infecting a series of mice with a dose of bacillus emulsion that in preliminary experiments had proved regularly fatal 3 days after subcutaneous injection. Emetin was administered by intra-peritoneal injection immediately before the injection of bacilli and subsequently at daily intervals until the animals succumbed. The results are shown in Table 6.

TABLE 6

ACTION OF EMETIN ON B. TETANUS (MULTIPLE DOSES OF EMETIN)

Mouse	Weight (Grams)	Dose of Emetin (Grams)	Equivalent in Grains per 60 K. or 132 lb. Body Weight	Number of Doses	Total Amount of Emetin Administered	Day of Death		
						1	2	3
1	20	.000381	15	2	.000762	—	+	
2	25	.000321	12	3	.000963	—	—	+
3	18	.000193	10	2	.000386	—	+	
4	17	.000145	8	2	.000290	—	+	
5	22	.00014	6	2	.00028	—	+	
· 6	24	.000102	4	3	.000306	—	—	+
7	25	.000054	2	3	.000152	—	—	+
8	20	.000021	11	3	.000063	—	—	+
9	18	Control	..	...	....	—	—	+
10	16	Control	..	...	....	—	—	+

In both experiments all the mice showed the characteristic symptoms of tetanus intoxication before succumbing. In the second experiment the death of several mice before the death of the controls may have been due in part to the toxic action of emetin.

In view of these results with relatively large doses of emetin we may conclude that the drug exerted no appreciable bactericidal action in vivo on the tetanus bacillus. We must state, however, that some soluble and preformed toxin may have been present, and probably was, so that the slight bactericidal action of emetin in vivo, as shown with the anthrax bacillus was overshadowed by it in the experiments with tetanus bacilli.

TRYPANOCIDAL ACTION OF EMETIN HYDROCHLORID IN VIVO

On Trypanosoma equiperdum.—In these experiments white rats were infected with trypanosomes by intraperitoneal injections of approximately known numbers after the method of Kolmer.[11] Twenty-four hours later emetin, dissolved in sterile salt solution, was administered intravenously in increasing doses according to body-weight. The rats were examined daily for trypanosomes in the peripheral blood; in this manner we hoped to detect any trypanocidal action which emetin might exert in vivo.

The strain of T. equiperdum employed kills white rats with regularity from 4 to 6 days after infection with the dose employed. In this manner the duration of life alone could be accepted as a criterion of trypanocidal influence among those rats receiving emetin.

[11] Jour. Infect. Dis., 1915, 16, p. 311; 17, p. 79.

The results of these experiments are shown in Table 7. The first 4 rats died within a few minutes after the injection had been made, as the result of the toxicity of the very large dose of emetin. In a general way it would appear that emetin had exerted a slight trypanocidal action in vivo—shown in Rat 6, which remained sterile for 12 days, and in the slight lengthening of the duration of life in Rats 5, 11, 12, 13, and 15.

Rat 6 had received a dose equivalent to 0.65 gm. (10 grains) for a 132-pound man, which was but slightly under the lethal dose for this animal; likewise, the remaining animals had received relatively large doses, so that we may state that the trypanocidal action of emetin in vivo is very slight, and in evidence only when the drug is given in large doses.

On Trypanosoma lewisi.—Somewhat similar results were observed in our experiments with T. lewisi. These were conducted in exactly the same manner as were the experiments just described. The results of one experiment are shown in Table 8.

The strain of T. lewisi used was slightly pathogenic, in that infected rats begin to die off about 20 days after infection with the dose employed in our experiments. In this series the animals died at very irregular intervals and among the animals receiving emetin no influence on the duration of life was apparent. It is probable that the life of Rat 1 was shortened by the large dose of emetin administered.

The trypanocidal influence of emetin in vivo, if the drug really possesses this action, is very slight indeed; in this experiment it was shown by only a slight retardation of the multiplication of trypanosomes in the peripheral blood.

AMEBACIDAL ACTION OF EMETIN HYDROCHLORID IN VIVO

Our experience with emetin on amebae in vivo is limited to the use of the drug, administered hypodermatically, in the treatment of amebic pyorrhea. Bass and Johns[12] were the first to advocate this form of treatment, reporting that with the subcutaneous injection of 0.032 gm. of emetin daily for from 1 to 3 doses endamebae disappeared from all lesions in the mouth in more than 90% of all cases. After 6 treatments they disappeared in 99% of cases.

While in our experience the endamebae are best attacked by the local use of emetin, we have noted the amebacidal action of the drug administered hypodermatically; similar reports have been made by others and when it is considered that even a dose of 0.016 to 0.065 gm.

[12] New Orleans Med. and Surg. Jour., 1914, 67, p. 456. Jour. Am. Med. Assn., 1915, 64, p. 553.

TABLE 7

Action of Emetin Hydrochlorid in Vivo on T. equiperdum *

Rat	Weight (Grams)	Dose of Emetin (Grams)	Equivalent in Grains per 60 K. or 132 lb. Body Weight	Results of Examination of Blood from Tail											
				24 Hr.	48 Hr.	72 Hr.	96 Hr.	5 Da.	6 Da.	7 Da.	8 Da.	9 Da.	10 Da.	11 Da.	12 Da.
1	101	.00428	40	Died at once											
2	80	.00342	40	Died at once											
3	72	.00154	20	Died at once											
4	98	.00212	20	Died at once											
5	98	.00107	10	—	Few	+	++	++++	++++	Died					
6	98	.00107	10	—	—	++	—	—	Few	Few	++	+++	+++++	+++++++	Died
7	100	.000856	8	—	Few	++	+++	++++	Died						
8	92	.000787	8	—	Few	Died									
9	96	.000616	6	—	Few	++	++	++++	Died						
10	103	.00063	6	—	Died										
11	96	.00041	4	—	Few	+++	+++++	++++	++++	Died					
12	87	.00037	4	—	Few	+++	+++++	++++	++++	Died					
13	70	.000149	2	—	Few	+++	+++++	++++	++++	Died					
14	78	.000166	2	—	Few	+++	+++	Died	++++						
15	80	.000085	1	—	Few	++	+++	++++	Died						
16	83	.000088	1	—	Few	++	Died								
17	80	Control		—	+	+++++	+++++	Died							
18	100	Control		—	++	+++++	Died								
19	92	Control		—	++	+++	+++++	+	Died						
20	96	Control		—	++	+++	+++++	++++							

* Infecting dose of trypanosomes, 140,000; single dose of emetin intravenously 24 hours after infection
Few = 1-2 in a field, or in every other field.
+ = about 5-10 in a field.
++ = about 10-20 in a field.
+++ = large numbers that may be roughly counted (not accurately).
++++ = very large numbers; cannot be counted.

TABLE 8

ACTION OF EMETIN HYDROCHLORID IN VIVO ON T. LEWISI *

Rat	Weight (Grams)	Dose of Emetin (Grams)	Equivalent in Grains per 60 K. or 132 lb. Body Weight	24 Hr.	48 Hr.	72 Hr.	96 Hr.	5 Da.	6 Da.	7 Da.	8 Da.	9 Da.	10 Da.	11 Da.	12 Da.
1	80	.00102	12	—	—	—	—	—	Died						
2	99	.00108	10	—	—	—	—	Few	Few	+	++	++	++	++	++
3	90	.000769	8	—	—	—	Few	Few	+	+	+	+	+	+	+
4	84	.000539	6	—	—	Few	—	Few	Few	+	+	+	+	+	+
5	92	.000411	4	—	Few	—	Few	Few	+	+	+	+++	+++	+++	+++
6	92	.000205	2	—	—	—	Few	Few	+	+	+++	+++	+++	+++	+++
7	77	.000164	2	—	—	—	Few	Few	+	++	+++	+++	+++	+++	+++
8	70	.000075	1	—	—	—	—	Few	+	++	+++	++++	++++	++++	++++
9	66	Control	..	—	—	—	Few	+	+	+++	++	++++	++++	++++	++++
10	88	Control	..	—	Few	Few	Few	++	++	+++	+++	+++	++++	++++	++++
11	90	Control	..	—	Few	Few	+	++	+++	+++	+++	+++	++++	++++	++++
12	80	Control	..	—	—	Few	+	++	+++	++	+++	++++	++++	++++	++++

* Infecting dose of trypanosomes, 150,000; single dose of emetin intravenously 24 hours after infection.

(¼ to 1 grain) each day for from 3 to 6 days represents a very high dilution of the drug in the body fluids of the patient, it is readily understood that emetin wields a very high and specific amebacidal action in vivo.

Similar results have been noted in the treatment of amebic dysentery and its complications, the success of the drug in the treatment of this disease having suggested its use in the treatment of amebic pyorrhea.[1]

CONCLUSIONS

Emetin hydrochlorid administered intravenously to rabbits in doses varying from 0.065 to 0.52 gm. (1 to 8 grains) per 132 pounds of body-weight exerted slight or no antiseptic or germicidal influence on a virulent culture of Staphylococcus aureus; abscesses developed in the internal organs of the majority of experimental animals.

Emetin hydrochlorid administered intraperitoneally to mice in doses varying from 0.065 to 0.52 gm. and 0.975 gm. (1 to 8 and 15 grains) per 132 pounds of body-weight exerted no appreciable inhibitory or germicidal action on anthrax and tetanus bacilli.

Emetin hydrochlorid administered intravenously to white rats, infected 24 hours previously by intraperitoneal injection with T. equiperdum and T. lewisi, in doses varying from 0.065 to 0.78 gm. (1 to 12 grains) per 132 pounds of body-weight, appeared to exert a slight trypanocidal influence, which was most apparent in the experiments with T. equiperdum.

Emetin hydrochlorid is highly and specifically amebacidal in vivo, and its curative effects in amebic infections is to be attributed practically solely to this action. While the drug has slight bactericidal powers in vitro under the conditions of prolonged contact with micro-organisms, and while this germicidal action may enhance the value of emetin in the treatment of amebic infections by local application, in the light of our experiments this bactericidal action is not in evidence in vivo.

These observations constitute additional evidence of the active rôle played by Endamoeba gingivalis, Gros, in the pathogenesis of pyorrhea alveolaris; improvement or cure of this disease with emetin by subcutaneous injection is to be attributed solely to its amebacidal action. In the treatment with local applications of the drug the beneficial results are to be ascribed in most part to this same influence, altho here there is reason to believe that the beneficial effects are, to some degree at least, due to a coincident bactericidal influence on the part of the drug.

THE NATURAL RESISTANCE OF THE PIGEON TO THE PNEUMOCOCCUS [*]

WITH PLATE 7

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INTRODUCTION

Attempts to determine the biologic factors which govern natural immunity to bacterial infection have too often been attempts to reduce the mechanism of resistance to terms of a single factor. Thus, only after an extended epoch of controversy between two schools, each seeking to explain natural immunity in terms of a single general factor, namely, body cells in the one instance and body fluids in the other, have we come to recognize, as, for instance, in the rôle of opsonins, the dependence of each of these factors on the other and the fallacy of attempting to eliminate either.

In those instances, also, in which a relatively high or low body temperature has been advanced as the basis of resistance, the tendency has been to emphasize this single feature to the exclusion of other factors.

Thus, Pasteur,[1] in his early study of the resistance of fowls to anthrax, concluded that the high temperature of the fowl (41 to 42 C.) effected a direct heat-destruction of the bacteria, and that this was the full explanation of the immunity. Later, however, Hess[2] and Wagner[3] showed that phagocytosis, which occurs at the normal high temperature of the fowl, is a very important factor in immunity to anthrax, and that the increase in susceptibility produced by depressing the temperature, as practiced by Pasteur, is due, at least in part, to a reduction in the phagocytic activity of the cells at the abnormal temperature. So that in this instance also, the simpler explanation which attributed resistance to a single factor, the temperature of the body, erred in eliminating at least one other factor of importance, namely, phagocytosis.

Indeed, it may be said that in general much of the lack of success in the ultimate analysis of natural immunity appears to have resulted from the exclusion of contributing factors in the overemphasis of a single factor. Fortunately, however, the intensive study in the general field of immunity during the last two decades, has done much to correct

[1] Bull. de l'Acad. de méd., 1878, 7, p. 140.

[2] Arch. f. path. Anat., 1887, 109, p. 365.

[3] Ann. de l'Inst. Paşteur, 1890, 4, p. 570.

this tendency and to enforce recognition of the fact that natural resistance to bacterial infection is an involved biologic reaction, not reducible to a single term, but embracing a multiplicity of factors highly interdependent and variously combined in complex and delicate adjustments. A survey of the field at this time indicates therefore that objective intensive study of individual instances of natural immunity with a view to identifying as many factors as possible there operating, may be preferred to the attempt to elevate to a position of prime importance a single factor by identifying the same in a large variety of instances.

It is the purpose of this paper to report observations concerning the distribution of pneumococci and the site of their destruction within an insusceptible host, the pigeon; and to deduce from this evidence the factors which are involved in the elimination of the bacteria, and to consider the relation of this elimination to the pigeon's high natural resistance.

Before advancing to the details of the investigation, it may be well to recall certain anatomic and physiologic features in which pigeons differ from rodents, the animals oftenest employed in laboratory experimentation.

(1) The body temperature of the pigeon is relatively high, ranging from 41 to 43 C. (106 to 109 F.). (2) The rate of metabolism is rapid. (3) The lymphatic system is relatively elementary and lacks a general distribution of lymph nodes; but nodules of lymphoid tissue are to be found in the parenchymatous organs, collected for the most part near the hilum. (4) The red corpuscles are nucleated. They are of relatively large size, measuring approximately 13 by 7 microns, and the ultimate blood capillaries are of correspondingly large caliber. (5) The white blood corpuscles conform in general to the types found in mammals, except that the cytoplasmic granules of the cells homologous to the neutrophil polymorphonuclear leukocytes of man, have the form of slender rods. These rods are often the length of two-thirds the diameter of the cell, and are closely packed throughout the cytoplasm. The affinity of these rods for acid dyes is great, and this has led to the confusing designation of the cells which they occupy as "crystalloid eosinophiles." This type of cell is not, however, in any way homologous to the type of blood cell commonly designated as the eosinophile in other species. The true eosinophile occurs in the birds also, but bears no direct relation to the so-called "crystalloid eosinophile" which, as just stated, is the homologue of the neutrophile of man. The cell under discussion is best designated as the crystalloid acidophile cell. So-called "fusiform bodies" or "ellipsoids" are present in birds' blood, closely resembling those found in amphibian blood. They appear as nuclei with a small amount of cytoplasm at either pole. The polar tabs of cytoplasm may be drawn out, or they may be blunt and vacuolated. The exact nature and the rôle of these structures are in dispute. They display a marked tendency to clump. (6) The liver and the spleen of birds contain large numbers of specialized endothelial cells, which, as I have shown

elsewhere,[4] perform the normal function of phagocytosis and digestion of red blood corpuscles. These cells, which I have designated hemophages, are shown in the accompanying drawing of the normal pigeon's liver (Fig. 1). In birds, the greater number of hemophages are active in the liver, the spleen appearing to be supplementary in this function. By virtue of a marked iron content, the cells referred to may be strikingly differentiated by Perl's Prussian-blue reaction.

The possession by pigeons of a high resistance to the pneumococcus is not in dispute. In my own experiments I have not been able, with any dose of various strains of the pneumococcus, to produce symptoms of sickness, and this accords with the results obtained by others. Explanations of this marked resistance have been numerous and varied, but for the most part based either on the high temperature of birds or on the phagocytic activity of leukocytes.

Tchistovitch[5] was among the earlier workers to place emphasis on the rôle of leukocytes in this immunity, while Strouse[6] more recently has given support to the view that phagocytosis is not a factor of importance and that the high temperature is the determining factor in the resistance of pigeons.

THE ACCUMULATION OF PNEUMOCOCCI WITHIN THE LIVER AND THE SPLEEN

A critical question in any analysis of natural immunity is that which concerns the actual site of the destruction of the bacteria, and the following experiments bear largely on this point. It was first sought to determine the distribution of injected pneumococci within the various organs and tissues of the pigeon. For this purpose 5 series of 20 pigeons each were injected by way of the leg vein with virulent pneumococci and the pigeons of each series killed in pairs after increasing intervals. The tissues were immediately fixed and later systematically examined microscopically for their content of pneumococci. The 5 series, which differed from one another only as to the number and the virulence of the organisms injected, coincided throughout as regards general results. The details here given are those relative to the group of 20 pigeons designated as Series B.

Each pigeon of this series received by way of the leg vein 2.5 c.c. of such an emulsion of pneumococci in 0.85% NaCl solution that each cubic centimeter represented the organisms washed from 4 twenty-hour blood-agar slants, the total dose in each instance representing, therefore, 10 such cultures. The particular pneumococcus employed in this series had been recovered 5 days previously from the heart blood of a man who had died of lobar pneumonia;

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[4] Internat. Monatschr. f. Anat. u. Physiol., 1914, 31, p. 543.

[5] Ann. d. l'Inst. Pasteur, 1904, 18, p. 304.

[6] Jour. Exper. Med., 1909, 11, p. 743.

it had been passed once through a mouse, which it killed in 26 hours. The 20 birds thus injected were killed 2 at a time after each of the following intervals: 10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 18 hours, 42 hours, 72 hours, 96 hours, and 112 hours. Tissue was taken at once from lung, liver, spleen, kidney, genitalia, pancreas, intestine, breast muscle, and bone marrow. The tissues were fixed in alcohol, formalin-Zenker, and Müller's fluid plus 5% mercuric sublimate; embedded in paraffin and sectioned to 4 microns. Proximal serial sections were treated with Gram's stain alone; with acid carmin followed by Gram's stain; and with Mallory's methylene blue and eosin method. The sections thus prepared from the several organs were systematically searched for pneumococci, the Mallory specimens serving as topographic controls. The findings in detail are as follows:

10-MINUTE PIGEONS

LUNG: Pneumococci free within the small vessels. An apparent increase in leukocytes within the small vessels; no organisms seen within leukocytes. LIVER: Organisms in the capillaries in fair numbers; many apparently within cells. SPLEEN: Organisms in great numbers chiefly in association with the walls of the small arteries emerging from follicles; none within the follicles; a few in large vessels. Several cells in the pulp cords contained a few organisms each. KIDNEY: Very few organisms seen, mostly within smaller vessels and a few apparently within cells of the blood stream. PANCREAS: A few groups of 3 or 4 organisms each within the smaller arteries. INTESTINE: A few organisms within the small vessels of the villi. BONE MARROW: A few organisms throughout, apparently in close association with blood channels containing erythrocytes; some apparently contained within cells.

No organisms were found in the genitalia or the breast muscle.

30-MINUTE PIGEONS

LUNG: Organisms frequently seen within cells of smaller blood vessels; none free in vessels. LIVER: A large number of organisms, some within cells. Number distinctly greater than at 10 minutes. SPLEEN: Groups of organisms seen along the line of small arteries; none in follicles; large number within cells. Number of organisms about the same as at 10 minutes. Many more than elsewhere except in the liver. BONE MARROW: Organisms fewer than at 10 minutes. KIDNEY: Occasional cells within small blood vessels, containing from 1 to 8 organisms; no free organisms. INTESTINE: A few organisms found in vessels of villi.

No organisms were found in the genitalia, muscle, or pancreas.

1-HOUR PIGEONS

LUNG: Organisms in capillaries, apparently within cells, but not all. The number about as before. SPLEEN: "Loaded" with organisms, but none in follicles, being limited almost exclusively to zone about small arteries. The few organisms in pulp cords apparently within cells. MARROW: A few organisms. LIVER: Large number of organisms within liver capillaries, apparently almost exclusively confined to cells, a single cell often containing 20 pneumococci. Distinctly more organisms than before. KIDNEY: Occasionally an intravascular cell containing from 1 to 5 organisms, but number small as compared with that found in liver and spleen. PANCREAS: A single cell with 3 organisms. INTESTINE: A few organisms in vessels of villi, all apparently within cells.

No organisms were found in genitalia or muscle.

3-Hour Pigeons

LUNG: Many intravascular crystalloid leukocytes, one clump of 30. Few organisms and those apparently within cells of blood stream. SPLEEN: Many crystalloid leukocytes in pulp cords. Large number of organisms throughout, most in zones about small arteries; none in follicles; many organisms apparently within cells of pulp cords. Etched and irregular forms numerous. BONE MARROW: Small number of organisms, most, apparently, within cells. LIVER: Many crystalloid leukocytes. Many organisms, for most part herded as if confined to cells of capillary walls. Many etched and irregular forms. KIDNEY: But 5 organisms seen in 5 sections and these within crystalloid leukocytes of larger vessels. PANCREAS: Three organisms found in 2 specimens.

No organisms seen in genitalia, muscle, or intestine.

6-Hour Pigeons

LUNG: Large number of crystalloids in capillaries. Number of organisms about the same as at 3 hours. Two crystalloid leukocytes seen with 3 and 4 organisms each. SPLEEN: Large number of crystalloid leukocytes throughout. Organisms present in goodly numbers; about the same number as at 3 hours, possibly fewer. Etched and irregular forms frequent. Almost all apparently within cells. BONE MARROW: Filled with crystalloid leukocytes. Few organisms present, for the most part within cells. LIVER: Many crystalloids in capillaries. Many organisms in clumps of 5 to 20, apparently contained within cells. Altogether slightly fewer organisms than at 3 hours. Etched and irregular forms. PANCREAS: Three crystalloid leukocytes seen, each containing from 8 to 10 organisms. INTESTINE: An occasional leukocyte seen containing 3 or 4 organisms.

No organisms seen in kidney, genitalia, or muscle.

18-Hour Pigeons

LUNG: Many crystalloid leukocytes in capillaries. Few organisms and these exclusively within crystalloid leukocytes. SPLEEN: Organisms fewer than previously; all within cells; frequently irregular in form and staining. LIVER: Goodly number of organisms but fewer than at 6 hours. Many surely contained within cells; the picture is distinct. Etched and granular forms are frequent. KIDNEY: Six crystalloid leukocytes found containing from 1 to 5 etched organisms each.

No organisms seen in genitalia, muscle, pancreas, intestine, or marrow.

42-Hour Pigeons

SPLEEN: Few organisms. All etched or granular, and apparently within cells. LIVER: Considerable number of organisms, but distinctly fewer than at 18 hours. Organisms appear to be herded within cells. Many swollen, etched, and granular forms.

No organisms found in marrow, kidney, lung, genitalia, breast muscle, pancreas, or intestine.

72-Hour Pigeons

SPLEEN: Few organisms, all within cells. Many swollen and etched forms. LIVER: Many organisms; but fewer than previously. Most organisms are etched and of irregular form, all apparently within cells.

No organisms seen in lung, marrow, kidney, intestine, genitalia, muscle, or pancreas.

96- and 112-Hour Pigeons

No organisms seen in lung, spleen, marrow, liver, kidney, intestine, genitalia, muscle, or pancreas.

From these reports it is seen that the injected pneumococci, far from being uniformly distributed among the several organs, were preferentially deposited in the liver and spleen. Aside from liver and spleen more pneumococci were encountered in the lung and the bone marrow than elsewhere, but even in these organs, the number was in no way comparable to that observed in the liver and spleen. The approximate relative numbers of organisms present in the several organs at the various time periods are given in Table 1.

TABLE 1

The Approximate Relative Numbers of Organisms Present in the Several Organs at the Various Time Periods

Organ	10 Min.	30 Min.	1 Hr.	3 Hr.	6 Hr.	18 Hr.	42 Hr.	72 Hr.	96 Hr.	112 Hr.
Lung..........	++	++	++	+	+	+	0	0	0	0
Spleen.........	+++	+++	++++	++++	+++	++	+	+	0	0
Bone marrow..	+	+	+	++	+	0	0	0	0	0
Liver..........	++++	+++++	++++ / ++++	+++ / ++++	++ / ++++	++++	+++	++	0	0
Kidney.........	+	+	+	0	0	+	0	0	0	0
Genitalia.......	0	0	0	0	0	0	0	0	0	0
Breast muscle..	0	0	0	0	0	0	0	0	0	0
Pancreas.......	+	0	+	+	+	0	0	0	0	0
Intestine.......	+	+	+	0	+	0	0	0	0	0

An analysis of Table 1 shows that at all the intervals from 10 minutes to 72 hours the liver contained many more pneumococci than did any other organ. The spleen ranked next, tho showing distinctly fewer pneumococci than the liver had shown. In other organs the numbers were relatively inconsiderable. The contrasts recorded were marked far beyond the fine quantitative differences such as might be caused by accidental variations in distribution, or by differences between the particular parts of the organs from which specimens were taken. Moreover, as stated previously, 4 similar series of inoculations gave corresponding results.

The rate of the differential localization of pneumococci is rapid; as early as 10 minutes after injection the accumulation of pneumococci in the liver and in the spleen was already distinctly apparent. The rate

of destruction of pneumococci within these organs is also rapid; after 72 hours both the liver and the spleen were found to be free of the organisms.

The significance of these findings is in the fact that the completeness of the accumulation of the pneumococci in the liver and the spleen, the promptness with which the localization occurs, and the rapidity of the destruction of the organisms so segregated, constitute an actually efficient process for the elimination of pneumococci from the circulating blood.

THE MECHANISM OF THE ACCUMULATION OF PNEUMOCOCCI WITHIN THE LIVER AND SPLEEN

The recognition of an extensive accumulation in the liver and the spleen of pneumococci introduced into the general blood stream, involves at once the question as to the mechanism by which this accumulation is effected. A histologic study of these organs has led me to the conclusion that phagocytosis by fixed tissue cells is the basis of this accumulation, to which, in the spleen, is added a supplementary factor in the filtering action of the modified vascular wall of an artery peculiar to that organ.

In the reports reference is made to the fact that the pneumococci within the liver and the spleen were often observed to be in considerable aggregates, as tho herded within the confines of a cell. Nevertheless, with the more usual histologic methods, it still remained a question whether the pneumococci were actually intracellular, and if so, in what type of cell they were included. To obtain more decisive morphologic evidence in this regard I developed a method which is essentially a combination of Perl's Prussian-blue reaction for iron, of Gram's stain, and of one or more counterstains. The introduction of the Prussian-blue reaction was suggested by the fact that the bacteria, where collected in clumps, were in close relation with masses of golden-yellow pigment. This pigment appeared the same as the iron-containing pigment which I have elsewhere shown to be constantly present within certain endothelial cells of the liver and the spleen of normal pigeons, and which results from an intracellular digestion of red blood corpuscles by those cells (hemophages). Moreover, in the normal pigeon, the iron-containing pigment when subjected to Perl's test sharply differentiates the containing cell by virtue of the Prussian-blue formed within the cell body. In view of these facts it appeared possible that

in the case of the injected pigeons, the close relation of pigment and bacteria might be due to the inclusion of both within the same cell, and that this relation might be made apparent by the sharp differentiation of the containing cell by the Prussian-blue reaction. Such proved to be the case and the method was most extensively employed according to the following formula :

Fix thin slices of tissue for from 18 to 24 hours in Müller's fluid plus 5% mercuric sublimate. Imbed in paraffin and section to 4 microns. Fix sections upon slide and immerse 10 minutes in equal parts of a 2% aqueous solution of potassium ferrocyanid, and of a 2% aqueous solution of hydrochloric acid, sufficient sodium chlorid being added to bring the combined solution up to 2%. Wash in 2% sodium chlorid and stain for 20 minutes in acid carmin. After washing hurriedly in 50% alcohol, stain by Gram's method, decolorization being effected in a mixture of toluol (2 parts) and anilin oil (1 part). Wash in toluol, and mount in Canada balsam.

Prepared by this method, the liver and spleen of the injected pigeons afforded a striking picture. As in the case of the liver and the spleen of normal pigeons, both the organs showed an extensive content of clearly differentiated cells possessing the distinct blue-green tone of the Prussian-blue iron reaction. These hemophages were somewhat more numerous, or at least more prominent, in the case of the injected birds than in normal birds. The cells were of the same type in both organs and in the liver were clearly recognizable as vascular endothelium of the venous capillaries. In the spleen the vascular relation was less evident, and no hemophages were found within the follicles ; in other words, they were confined to the pulp cords. Wherever present, the hemophages contained ingested red blood corpuscles, or products of their digestion, and in the liver the inclusions produced a marked bulging of the cells into the lumen of the vessel. From the point of view of the present investigation, however, the most striking phenomenon observed in such specimens was the inclusion by the hemophages of practically all pneumococci injected (Figs. 2 and 3). With the sharp differentiation of the cell body of the hemophages by the Prussian-blue reaction, all doubt concerning the intracellular presence of the organisms was eliminated. Pneumococci were seen in most of the hemophages, and in a given hemophage in great numbers. In the 4-micron section of a hemophage, not infrequently 50 pneumococci were to be counted, this indicating that the content of the total cell was several hundred organisms. In many cells the organisms were distributed rather uniformly throughout the cytoplasm, while in others distinct vacuoles containing from 10 to 30 pneumococci were frequently

observed. The contained organisms displayed in many instances morphologic changes resulting from intracellular digestion—etched, swollen, and granular forms being the most frequent.

With the fact established that the hemophages of the liver and the spleen so extensively take up pneumococci, a detailed survey was made of tissue of these two organs taken from all pigeons of Series B, with especial reference to the relative numbers of the organisms actually contained within the hemophages. The results showed that throughout the series practically all pneumococci accumulated within the liver were contained within the hemophages. Occasionally 1 to 3 pneumococci could be found apparently free in a lumen or within a crystalloid acidophile leukocyte of the circulating blood, but this occurrence was rare and limited almost exclusively to pigeons killed within one-half hour of their injection. Even in such instances, however, the number of organisms not included within hemophages would not, in a total averaged-sized microscopic preparation, equal the number of organisms often seen within a single hemophage of the same specimen.

It thus appears that the marked accumulation of pneumococci in the liver subsequent to their intravenous injection is due exclusively to the extensive engagement of the organisms by that type of fixed phagocyte which has for its normal function the destruction of red blood corpuscles.

In the spleens of the series, the hemophages were seen to play the same rôle as in the liver, the pneumococci ultimately being accumulatd within these cells and there digested. Cell for cell, the hemophages of the spleens contained as many pneumococci as did those of the livers. The absolute number of hemophages was, however, as in normal pigeons, distinctly smaller. Moreover, the primary localization of the pneumococci within the spleens was not due, as in the livers, exclusively to the action of the hemophages. A mechanical filtration of the organisms by the walls of certain of the blood vessels was found to be an important factor in the rapid accumulation of the pneumococci within this organ, and the structure of these vessels therefore requires consideration in detail. The small vessels emerging from the follicles of the bird's spleen consist of a delicate endothelial intima surrounded by a double or even triple layer of spherical cells sharply differentiated from other tissue elements and supported in a delicate reticulum meshwork. This double or triple layer of cells constitutes a broad zone about the lumen of the vessel and the wall thus constituted bears but slight resemblance to any other vessel wall with which I am acquainted.

The sharpness of the demarcation of the outer margin of the layer, the uniformity of its area of cross section, and the characteristic staining reaction of the constituent cells reveal it, however, as a vascular coat supporting and supplementing the intima of the vessel in question. In cross section this vascular coat appears of relatively great thickness but of loose texture. The general structure of such vessels in cross section is shown in Figure 4.

With this brief statement concerning the structural detail of the splenic vessels in question, it may be stated that they serve a distinct function as filters under the conditions of the experiments here recorded, and operate to accumulate promptly intravascular pneumococci within the spleen. The evidence for this is seen in the distribution of the pneumococci within the spleen soon after injection. In the 10-minute spleens, for instance, practically all pneumococci observed are found between the cells of the vascular coat. No organisms are seen in the follicles and but very few are at this time found within the hemophages of the pulp cords. In a 4-micron section of a single vessel of this type, 10 minutes after injection, it is frequently possible to observe from 50 to 100 pneumococci definitely limited to the vessel wall (Fig. 4). The pneumococci are not intracellular. They are distinctly interstitial and are not clumped. The vascular zone is free of hemophages as are also the follicles. At this period a few intravascular crystalloid leukocytes may be found containing from 1 to 4 pneumococci and an occasionally free pneumococcus may also be seen in the blood stream. In 30-minute spleens the number of pneumococci within the zones is much increased, but a considerable number of organisms also have emigrated to the pulp cords in the vicinity of the zones, and have been engulfed by hemophages there contained. In 1-hour spleens also, there are many pneumococci between the cells of the vessel walls, but, in addition, they are now found widely distributed throughout the pulp cords, being contained within hemophages. In spleens from 3-hour and 6-hour pigeons the findings contrast with those in the earlier animals in that the greater proportion of organisms is now found within the hemophages of the pulp cords, and relatively few are retained within the vascular zones. In the pigeons killed 18 hours or longer after injection, the zones are free from pneumococci, the organisms having been transported completely through the vessel wall and engulfed by hemophages of the pulp cords.

Surveyed from the point of view of function this distribution of the pneumococci within the spleen after various intervals leads to the conclusion, (1) that pneumococci which enter the spleen by way of its closed vascular system rapidly leave this system, being transported into, and eventually through, the loose vascular wall of certain modified arterioles whose interstices allow the free outward passage of plasma but not that of the formed elements of the blood; (2) that the interstices between the cells constituting the great bulk of the vascular wall in question are of such minute size as to allow but a retarded transportation of pneumococci through them, the tissue thus operating as a partial filter accumulating the organisms within its area; and (3) that having thus gradually been washed through the vessel wall, the pneumococci are brought into contact with the hemophages of the pulp cords and by these are ingested and digested as in the liver. Thus, vast numbers of pneumococci are rapidly filtered from the plasma by a mechanism peculiar to the spleen, to be ultimately ingested and destroyed, however, by the same kind of phagocytic cell that we find in the liver. In both organs, therefore, the ultimate localization and destruction of the injected organism is within fixed phagocytes.

THE RELATION OF FIXED PHAGOCYTOSIS TO THE IMMUNITY OF THE PIGEON

I have shown that pneumococci introduced into the blood stream of pigeons are rapidly localized in the liver and the spleen and are there destroyed within fixed phagocytes. This demonstration does not prove that the immunity of the pigeon is based on this phagocytosis alone. The extent of the process, however, does indicate that this phagocytosis is a factor of great importance and possibly even the determining factor in the immunity. At best the degree of this importance can be determined only approximately, in view of the incompleteness of our present knowledge as to the many additional factors which may contribute to resistance. But so far as an estimate can be made, it is to be gained from a comparison of the efficacy of the fixed-tissue phagocytosis with that of such factors as wandering-cell phagocytosis, the antibacterial properties of the body fluids, and the influence of the temperature of the host.

In making such a comparison it should be borne clearly in mind, relative to fixed-tissue phagocytosis, that the significant feature is not the determination that a phagocytosis occurs but rather the demonstration of the great rapidity and extent of its occurrence. It is not diffi-

cult to conceive a fixed-tissue phagocytosis which, altho demonstrable, might involve so few organisms or proceed so slowly as to be practically negligible with respect to the elimination of the infecting organisms. In the present instance, however, both the extent and the rapidity of the phagocytosis are extreme: not a few, but practically all pneumococci introduced into the blood stream are taken up by the hemophages and this within a few hours. The phagocytosis, moreover, is a phagocytosis of living organisms, as shown by the fact that the liver is highly infectious up to 6 hours after injection, even after vascular washing to the point where the fluid recovered is slightly, and at times not at all, infectious. It is with such a phagocytosis that other factors must be compared.

Wandering-cell phagocytosis is clearly not a considerable factor in the immunity of the pigeon to the pneumococcus. This point I wish to emphasize the more because of the frequent confusion of fixed phagocytes with wandering cells, and because of the rather general conception that phagocytosis in relation to immunity coincides with phagocytosis on the part of wandering cells. Thus, Metchnikoff[7] expressed the view that the Kupffer cells — the hemophages of the liver — are to be regarded as wandering cells, namely, phagocytic white blood corpuscles which are somewhat delayed in the capillaries of the liver. The sharp differentiation of these cells in the pigeon's liver which I have obtained with the Prussian-blue reaction, shows conclusively, however, that these cells are not leukocytes but fixed cells. Their phagocytosis therefore is not to be confused with that accomplished by wandering cells.

The injection of pneumococci into the blood stream of pigeons produces a leukocytosis. This leukocytosis is almost exclusively an increase in the crystalloid acidophiles — the homologues of the neutrophiles in man. The normal average leukocyte count in pigeons is approximately 10,000 per cubic millimeter and there is usually no increase in this number for 1 hour after injection of pneumococci. In from 1 to 2 hours after injection, however, there is the commencement of a very sudden increase in the leukocytes, which may result in a content of 40,000 per cubic millimeter during the fourth or fifth hour. Between the tenth and twentieth hours from 50,000 to 100,000 per cubic millimeter is common. Soon after this period, however, the leukocytosis decreases rapidly so that at 40 hours, or even earlier,

[7] L'immunité, 1901, p. 80.

the leukocyte count is that of the normal animal. This cycle is the one observed following an intravenous injection of living virulent pneumococci.

Concerning the occurrence of a marked leukocytosis, therefore, there is no doubt; but equally striking is the almost complete lack of phagocytosis by the leukocytes. The results obtained both from blood smears and from the fixed tissues taken at various periods, show that of the vast number of leukocytes present, but very few contain pneumococci and then only in small numbers. (Unless the decolorization in Gram's method is extensive, the rodlike granules of the crystalloid acidophiles may so far simulate bacteria as to lead to the interpretation of phagocytosis when none exists. No such confusion occurs, however, when the extraction of the gentian violet is accomplished by a mixture of toluol and anilin oil.

Altogether the phagocytosis by wandering cells is very slight, and compared with the phagocytosis by the hemophages is so insignificant as to be negligible as a factor in the destruction of pneumococci and in the resistance of the host.

The evaluation of the rôle of the body fluids in the resistance of the pigeon to pneumococci can not be made with definiteness. Within the host no direct determination of the antibacterial action of these fluids can be effected apart from many complicating factors, and the analyses obtained in vitro carry no guarantee that the properties displayed there are more than remotely related to those which actually obtain within the animal. So far as in-vitro experiments are of value, however, they give no evidence of a distinct antibacterial action on the part of the blood serum — a conclusion reached also in the work of Strouse.[6]

Pneumococci subjected to the action of the serum in vitro do not show a diminished viability or pathogenicity beyond that produced by similar treatment with the sera of highly susceptible species. In fact, on agar slants with 2 c.c. of fresh serum at the bottom of the tube, the growth of pneumococci is most abundant at the line of contact betwen the serum and the agar; and pneumococci injected into mice 2, 4, and 8 hours after suspension in pigeon's serum show as great a killing power as when suspended similarly in salt solution or in the sera of the susceptible species—mouse, man, and rabbit.

In regard to the opsonic power of pigeon's serum there is also no evidence of any marked action, and here again my results coincide with

those of Strouse.[6]   The lack of any considerable phagocytosis of
injected pneumococci by the leukocytes in the circulating blood stream,
in itself, shows the absence of an opsonizing power, effective in relation
with these cells.   Tests in vitro of the opsonic value of the serum in
relation to the pigeon's own leukocytes are, in my opinion, of no value
whatever because of the extreme fragility of these leukocytes when
removed from the animal.   (So delicate are the crystalloid acidophile
leukocytes that, removed from the body, they rapidly go to pieces even
in the pigeon's own serum.   They cannot be suspended in the usual
diluents employed in leukocyte-counting, but for this purpose must be
preserved by rapid fixation, preferably in warm osmic acid.   Those
structures counted as leukocytes in the usual determinations of bird's
blood are for the most part nuclei of hemolysed erythrocytes; hence
the great discrepancies between the figures of various workers—ranging
from 10,000 to 50,000 per cubic millimeter.)   Tested with the leu-
kocytes of other species (mouse, man, guinea-pig, and rabbit), pigeon's
serum shows no definite opsonic power.   Altogether, then, such results
as have been obtained by test-tube methods fail to show that the blood
serum of the pigeon possesses a peculiar content of antibacterial sub-
stances which might operate to determine the immunity under
discussion.

The relatively high temperature of the pigeon requires careful con-
sideration.   Influenced by the early work of Pasteur on fowl anthrax,
and by the fact that the temperature of pigeons coincides approxi-
mately with the maximal surviving temperature of the pneumococcus
on artificial media, a school of workers has contended that the immunity
of that animal is due to a direct heat-destruction of the bacteria within
the host.   In support of this view investigations have been advanced
to show that phagocytosis is lacking, and the deduction made that
because of this lack, heat-destruction is the presumable factor which
confers the resistance.   The most explicit data in support of this view
are those furnished in the work of Strouse.[6]   In regard to this conten-
tion, the determination which I have made of an extensive fixed-tissue
phagocytosis possesses a direct bearing.   The results of my experiments
show not only that phagocytosis is not lacking, but that its occurrence
is of such magnitude as to demand its consideration as the major factor
contributing to the pigeon's immunity.

As indicated at the commencement of this article, I do not, in empha-
sizing the rôle of phagocytosis, wish to attempt to exclude possible con-

tributing factors, and among them temperature. On the other hand, the exalted position given temperature on the basis of negative findings as to phagocytosis is most certainly invalidated by the present demonstration that an extensive phagocytic destruction of pneumococci does occur, and further considerations relative to this phagocytosis indicate that the influence of the high temperature is at most subsidiary and indirect.

Among such considerations is the fact that the phagocytic destruction of the bacteria is accomplished in distinctly less time than that required for heat destruction at the maximal temperature of the pigeon. Ninety-six hours at 43 C. does not destroy pneumococcus cultures, but, injected into the blood stream of the pigeon, several billions of pneumococci are taken up within 10 minutes, and totally engaged and destroyed in about 72 hours.

A second indication that the resistance of the pigeon is not primarily due to high temperature is seen in the fact that 2 strains of pneumococci acclimated to 42.4 C. for several months, showed no pathogenicity or increased viability when injected into pigeons, altho rendered lethal for white mice and rabbits by passage. Their phagocytic destruction was in all ways similar to that of the usual cultures grown at 38 C.

The chief contention which I wish to make, however, is not that the temperature of the body, or indeed the action of body fluids, may be eliminated from all considration as contributory factors, but rather that phagocytosis does occur, and occurs in such magnitude as to demand its acceptance as a factor of major, if not determining, importance in the immunity displayed by the pigeon to the pneumococcus.

In the bulk of my experiments the organisms were introduced directly into the circulating blood stream. Were the action of the hemophages confined to the destruction of organisms so introduced. the general significance of their activity in the total immunity of the host might well be considered limited. This is not the case, however, for subsequent to their inoculation by other channels, pneumococci rapidly gain entrance to the blood stream, and, borne to the hemophages, are destroyed as are those injected intravenously. In the case of intraperitoneal injections the rapidity with which the organisms reach the general circulation, and thereby the hemophages, is most striking.[8] Whether it be the unusual size of the lymphatic spaces and vessels leading from the peritoneal cavity, or the absence of interposed nodes in their course, has not been determined, but the fact is established that

[8] Berry and Melick: Jour. Immunol., 1916, 1, p. 47.

pneumococci injected into the abdominal cavity reach the hemophages of the liver and spleen, and are there destroyed, in a period but slightly greater than that occupied in the destruction of organisms introduced directly into the blood stream.

CONCLUSIONS

In the pigeon, a species insusceptible to the pneumococcus, the infecting organisms are rapidly withdrawn from the general blood stream and localized in the liver and the spleen.

In both these organs the ultimate localization of the pneumococci is within a type of fixed phagocyte—the hemophage—common to both organs, and having for its normal function the destruction of red blood corpuscles.

This phagocytic destruction of the pneumococci by hemophages is so extensive and so rapid as actually to constitute an important, if not indeed the determining, factor in the establishment of this instance of natural immunity.

EXPLANATION OF PLATE 7

FIG. 1. Section of the normal liver of a pigeon showing differentiated hemophages. I=nuclei of hemophage. II=ingested erythrocyte. Camera lucida. Zeiss ocular 4, objective 2 mm. oil. × 820.

FIGS. 2 AND 3. Sections of the livers of pigeons killed one-half and one hour, respectively, after injection of pneumococci. Content of ingested pneumococci within hemophages. I=nuclei of hemophages. II=ingested erythrocytes. III=nuclei remnants of digested erythrocytes. Camera lucida. Zeiss ocular 8, objective 2 mm. oil. × 1600.

FIG. 4. Section of the spleen of a 10-minute pigeon showing pneumococci accumulated within the vascular zones. Camera lucida. Zeiss ocular 6, objective 2 mm. oil. × 1050.

PLATE 7

1.

2.

3.

4.

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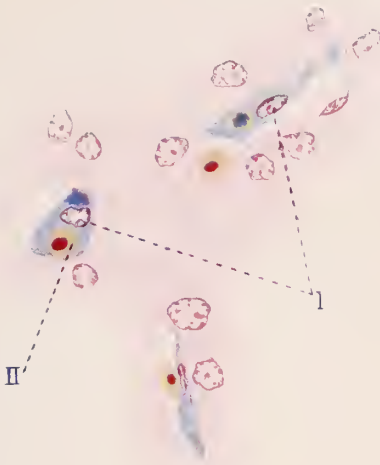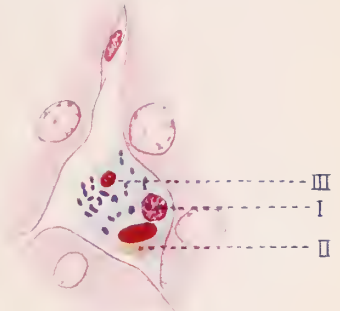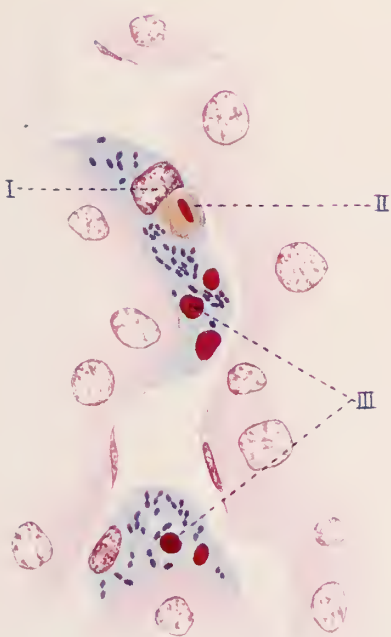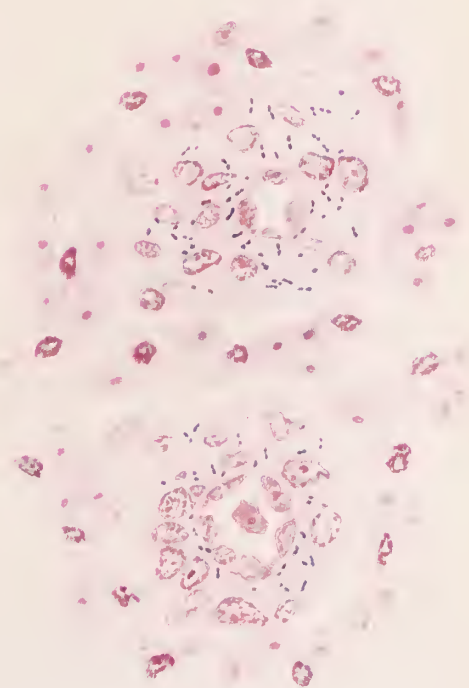
THE SANITARY CONTROL OF SWIMMING POOLS *

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Within the past decade the indoor swimming pool has become an important adjunct to Y. M. C. A.'s, colleges, universities, city clubs, and diverse other organizations interested in the improvement of our physical welfare. In the larger cities there is considerable agitation for the installation of tanks in the public schools. In a hotel now under construction there is to be an 80-foot plunge bath for the use of guests.

The possibility of transmitting infectious diseases through pools is obvious. Manheimer[1] cites several striking instances of infections among bathers, and following the classification of Atkins, divides such infections under 3 heads: (1) intestinal, (2) eye and ear, (3) venereal. Respiratory disease might be added as a fourth. Lewis[2] remarks on the presence of grippe, colds, pneumonia, and sore throats among the frequenters of the pool at Northwestern University, and similar observations are recorded by Burrage[3] at Purdue, Whipple[4] at Brooklyn, Polytechnic, etc. During the two years' service of a tank at the Iowa State College, there has been no epidemic of any kind traceable to the pool, but there have been sporadic complaints of colds and sore throats. The danger of infection in pools has probably been over emphasized. Nevertheless, we should regard the swimming tank as a potential vehicle of infection and take the necessary measures to control its sanitary condition.

Since the beginning of this investigation articles have appeared by Manheimer,[1] and by Clark and Gage.[5] Their conclusions as to the efficacy of disinfection with hypochlorite are so at variance that the question of the proper control of pools is still an open one.

THE SWIMMING POOL OF THE IOWA STATE COLLEGE

The tank is located in a large well-ventilated and well-lighted room. The walls of the room are of white-enameled brick.

[1] Jour. Infect. Dis., 1914, 15, p. 159.
[2] Eng. News, 1911, 65, p. 689.
[3] Ibid., 1910, 63, p. 740.
[4] Municipal Jour. and Engineer, 1911, 30, p. 577.
[5] Ann. Rep. Mass. State Bd. of Health, 1912, p. 347.

Construction.—The pool has a capacity of 90,000 gallons. It is 60 ft. by 30 ft., and the floor slopes so as to give 3 feet of water at the shallow end and from 7 to 8 feet at the deep end. The tank is of concrete with sides and bottom of white-enameled brick. Running around its top is a 6-inch gutter, or trough, which is connected directly with the sewer drains. This serves the triple purpose of an overflow gutter, an expectoration trough, and a handrail. The gutter is bounded by a ledge of white terra cotta about 1 ft. wide. Extending around, 4 inches below this ledge, is a 5-ft. concrete walkway so constructed as quickly to carry off all splash water, etc., into the sewer. Just off the walkway, on one side of the pool, are a shower room, a toilet, and a dressing room.

Operation and Supervision.—During school sessions, the pool is open from 4 to 6 p. m., and on 2 evenings a week. Occasionally it is used for class work between 10 a. m. and noon. By means of floor talks and printed notices posted in conspicuous places, the necessity and desirability of cleanliness are constantly brought to the attention of the bathers. Entrance to the pool is through the shower room, where an instructor is stationed to see that each person takes a proper shower with liberal use of soap. Even under these conditions of strict supervision it is doubtful whether an adequate bath is really taken by the majority of the bathers. The average attendance during Fall, Winter, and Spring is about 300 a week. During Summer about 500 people a week use the tank. Supervision is not so strict, since no medical examination is required of summer students and about half the frequenters are children under 14 years.

Water.—The water is obtained from 2 deep wells. It is of excellent quality bacterially, rarely having a count above 10 at body temperature, while tests for B. coli are uniformly negative in 100-c.c. samples. The mineral content, particularly iron, is extremely high. The iron is removed by mechanical filtration. Alum was employed at first, but discontinued. After filling the pool, the water is subjected to continuous filtration. The water is drawn through 2 pipes near the bottom at the deep end, pumped onto the filters, and returned to the pool through 2 pipes at the shallow end. About 50,000 gallons, or approximately one-half the capacity of the tank, are filtered daily. Occasionally fresh water is added to make up loss due to usage and leakage. The water is heated by steam forced in through 2 openings at the deep end of the pool, about 3 feet below the surface.

METHODS OF ANALYSIS

A review of the literature shows a lack of uniformity in methods of bacterial analysis of pools.

Ravenel[6] and Tully,[7] respectively, employed the count in gelatin at 20 C. and in litmus lactose agar at 37 C. For B. coli they used dextrose broth followed by confirmatory tests. Lewis[2] incubated for 72 hours at room temperature. Norton[8] plated on agar at 20 C. and on litmus lactose agar at 37 C. Manheimer[9] also plated on agar at room temperature, but employed plain agar at body temperature. He used the Jackson bile medium for B. coli. Another combination of media was gelatin at 20 C. and plain agar at 37 C., used by Thomas.[10] Clark and Gage[5] incubated litmus lactose agar and lactose

[6] Am. Phys. Edu. Rev., 1912, 17, p. 684.
[7] Am. Jour. Pub. Hyg., 1912, 2, p. 186.
[8] Am. Jour. Pub. Health, 1914, 4, p. 1016.
[9] Am. Phys. Edu. Rev., 1912, 17, p. 669.
[10] Jour. Ind. and Eng. Chem., 1915, 7, p. 496.

bile at 40 C. for the body temperature count and for B. coli determinations, respectively.

As a result of this lack of concordance in analytic methods, comparisons of the data from different pools are of little value. The necessity of some standard method of pool analysis is obvious.

In the study here reported, samples were collected once or twice a day for the first month and later at less frequent intervals. Unless otherwise noted, all samples were taken at the deep end, from 12 to 18 inches below the surface. Plates were poured within an hour after collection, and incubated at 37 C. for 24 hours. Both plain and litmus lactose agar (beef extract media) were employed. There were considerable and irregular variations between counts on plain agar and those on litmus lactose agar; finally, plain agar at 37 C. was adopted for the total count. Presumptive tests for B. coli were made by inoculating 1 c.c. into each of 5 Durham fermentation tubes containing lactose peptone bile. In some instances 10-c.c. and 0.1-c.c. samples were also tested for gas-formers.

EFFECT OF CONSTANT FILTRATION WITHOUT DISINFECTION

Distribution of Bacteria in Pool.—Samples taken from different parts of the tank showed some differences in bacterial content, but these variations were not of sufficient magnitude to be of any sanitary significance. The water from the filters is somewhat cooler and denser than that in the pool. A stratification might therefore result with a somewhat purer water at the bottom. While in use, the pool contents are reasonably well mixed, but during the long quiescent periods (20-22 hr.) depended upon for purification, stratification would tend to decrease the efficiency of the filtration process, for only the purer bottom strata would be passing through the filters.

Table 1 shows that some stratification does result. This could be avoided by changing the heating system in such way as to make the water entering the pool somewhat warmer than that already present in the tank.

TABLE 1

Distribution of Bacteria Near the Surface and Near the Bottom of the Pool After Continuous Filtration for 6 Days, Pool Not in Use

Location	Bacteria per c.c.	
	1 ft. Below Surface	Bottom
Shallow end...	1800	2300
Side..	2500	1650
Deep end, middle...	3200	1800
Deep end, corner..	3400	1400
Average..	2725	1780

Purification by Filtration.—Comparison of the bacterial content immediately after use with that from 18 to 20 hours later showed that the former was usually considerably higher. The reduction may be attributed to filtration, sedimentation, and possibly to the germicidal action of sunlight. During 20 weeks of operation with continuous filtration and no disinfection, the average bacterial purification was 60%. In a few instances, however, the count increased (see Table 3 and Chart 1). In consideration of the fact that only about half the water passes through the filters in 24 hours, the purification obtained (60%) would indicate that there was no multiplication of bacteria capable of developing on beef extract agar at 37 C.

Effect of Temperature on Bacterial Content.—There is some difference of opinion as to the proper temperature for plunges. From the point of view of the bacteriologist a pool is successfully operated if the bacterial count is low. This may be materially aided by a low temperature. The physical director, on the other hand, gages success by the attendance. This is favorably influenced by warmer temperatures. Of 5 physical directors in the Middle West, 1 reports for his pool a temperature of 70-72 F., another 76-83 F., and 3 maintain a temperature of 78 F. This is in marked contrast to the published reports of 7 pools in Massachusetts, Wisconsin, and Connecticut, in which the temperatures recommended are 70-72 F., except in one instance where 70-74 F. is prescribed.

At the Iowa State College the water is heated by forcing live steam into the tank. During the first few months it was extremely difficult to maintain a constant temperature. This variation, from below 70 F. to well above 80 F., afforded an opportunity to correlate temperature with bacterial content. Chart 1 shows a rather marked parallel between these two factors.

In Table 2 is indicated the effect of temperature as observed in a series of about 100 analyses. As practically all published reports recommended temperatures below 74 F. this temperature was selected for computing the table.

TABLE 2

RELATION BETWEEN THE TEMPERATURE AND THE BACTERIAL CONTENT OF A POOL

Bacteria per c.c. (Agar 37 C.)	Percentage of Samples		B. coli in 5 c.c.	Percentage of Samples	
	Below 74 F.	74 F. and Above		Below 74 F.	74 F. and Above
0-100..........................	7.7	2.9		42.9	21.2
101-500........................	38.5	7.4	Less than 1	42.9	21.2
501-1000.......................	3.9	5.9	1	7.1	24.2
1001-5000......................	30.7	38.1	2	10.8	10.6
5001-10,000....................	3.8	22.2	3	21.5	12.2
10,001-50,000..................	7.7	20.6	4	3.5	10.6
Over 50,000....................	7.7	2.9	5 or more	14.2	21.2

Table 2 shows that B. coli was absent from 42.9% of the 5-c.c. samples when the temperature was below 74 F. as compared with 21.2% when the temperature was above 74 F. Of all samples taken when the temperature was below 74 F., 80.8% had bacterial counts below 5000,

and 50% below 1000, while of samples taken with temperatures above 74 F., 54% contained less than 5000, and only 16.2% less than 1000 bacteria per c.c. We conclude from these results that, without the use of disinfectants, it becomes more difficult to maintain a swimming tank in good sanitary condition as the temperature rises above 74 F. To avoid chills the temperature of the room should be 4 or 5 degrees above that of the water in the pool.

In the lower part of Chart 1, the bacterial content of the pool before bathing is indicated by a continuous line; the content after bathing, by a broken line. In the upper part of the plot, the relative numbers of B. coli in 5-c.c. samples, before and after the tank was used, are shown by full and broken lines, respectively. On this plot are shown the analyses of all samples from October 27, when the tank was put into operation, to February 1, when it was emptied and cleaned.

Several facts are clearly demonstrated:

1. There is a marked fluctuation in the temperature, the total bacterial count, and the number of B. coli on different days. A rise in temperature is usually accompanied by an increase in bacteria.

2. From November to February there is a gradual, but fluctuating, decrease in the total number of bacteria, but the number of gas-formers increases. During the first 6 weeks B. coli was found only occasionally in 5 c.c. before use of the pool. After this period (with one exception) B. coli was constantly present in 5 c.c. It seems as if the organism had become adapted to its new environment.

3. On November 6 there was a marked drop in the number of bacteria, the count remaining low for about a week. During this period there was a considerable amount of sediment. This sediment became somewhat distributed throughout the tank during use, and in settling probably effected considerable purification by carrying down the bacteria mechanically. Manheimer noted that in a pool with a rather turbid water the bacterial content was relatively low.

4. The increase in B. coli after use of the pool is more striking than the relative increase in the total count.

EFFECT OF CONSTANT FILTRATION AND PERIODIC DISINFECTION WITH CALCIUM HYPOCHLORITE

Constant filtration alone, will at times keep the bacterial count low, but the preceding study indicates that this procedure cannot be relied on to maintain the pool in a sanitary condition. The danger of infec-

TABLE 3

BACTERIAL ANALYSES OF THE WATER IN THE SWIMMING POOL OF THE IOWA STATE COLLEGE, EMPLOYING CONTINUOUS FILTRATION WITH NO CHEMICAL DISINFECTION

Date	Time*	Bacteria per c.c. (Agar 37 C.)	B. coli in 5 c.c.	Tempera- ture, F.	Remarks
1913					
10/27	B2	2,700	0	..	
28	A1	11,000	2	68	
29	B	73,000	1	76	Circulation of water begun
29	A	20,000	4	78	
30	B	64,000	3	72	Pump stopped at 12 m.; pump started at 6 p.m.
30	A	60,000	3	63	
31	B	17,000	0	62	Alum discontinued
11/ 1	A	18,000	0	68	
3	B	4,400	0	68	
4	B	10,000	0	72	
6	A	135	2	72	Pool turbid, with sedi- ment on bottom
7	B	63	0	72	
7	A	269	3	68	
8	B	74	0	74	
10	B	18	0	..	
10	A	68	1	74	
11	B	110	1	74	
11	A	120	1	74	
14	A	330	2	74	Sediment removed
15	A	9,000	1	74	
17	B	6,500	1	76	
17	A	2,000	3	76	Pump and filtration stopped
18	A	2,600	4	78	
19	A	35,000	3 .	78	
20	A	16,000	1	74	
24	B	22,000	0	76	
24	A	19,000	4	76	
26	A	......	2	77	Pumps and filters started
29	A	16,000	1	78	
12/ 1	B	4,000	1	77	
2	B	2,300	0	75	
3	A	8,800	2	78	
3	B	18,500	1	78	
3	A	32,000	3	80	
4	A	16,000	2	86	
8	B	1,150	0	83	
9	B	2,200	0	78	
10	A	8,000	1	75	
11	A	5,500	1	76	
15	A	565	0	•	
16	B	......	0	..	
16	A	12,500	1	..	
17	B	......	0	..	
17	A	1,300	3	70	
18	B	......	1	..	
18	A	1,200	5†	..	
19	B	......	5†	..	
19	A	7,800	5†	77	
20	A	9,300	5†	74	Pool not in use Dec. 20 to Jan. 12
1914					
1/15	B	3,000	0	70	
15	A	1,600	2	73	8 inches of raw water added
16	B	625	4	68	

* A1 = sample taken during use or immediately after.
  B2 = sample taken before pool was opened for the day.
  † All five 1-c.c. samples tested were positive. There were therefore 5 or more gas-formers in 5 c.c.

TABLE 3—*Continued*

BACTERIAL ANALYSES OF THE WATER IN THE SWIMMING POOL OF THE IOWA STATE COLLEGE,
EMPLOYING CONTINUOUS FILTRATION WITH NO CHEMICAL DISINFECTION

Date	Time*	Bacteria per c.c. (Agar 37 C.)	B. coli in 5 c.c.	Tempera-ture, F.	Remarks
16	A	11,500	5†	73	
17	B	7,100	5†	78	
19	B	13,000	1	81	
20	A	475	5†	80	
21	B	2,500	4	78	
22	B	3,100	4	78	
22	A	2,000	4	77	
23	B	4,900	4	77	
23	A	4,300	5†	76	
24	B	950	4	76	
1914					
26	B	51	1	74	
26	A	120	5†	..	
27	B	185	3	72	
27	A	280	5†	72	
29	A	425	5†	72	
30	B	400	1	72	
30	A	450	5†	72	Pool emptied Feb. 5, sides washed, and pool refilled Feb. 9
2/11	B	450	0	56	
13	B	4,500	0	64	
16	B	32,000	0	76	
16	A	83,000	2	74	
17	A	27,500	5†	74	
18	B	7,500	5†	77	
18	A	21,100	5†	74	
19	B	8,000	5†	74	
19	A	4,000	5†	74	
20	B	1,400	5†	74	
23	B	4,600	5†	74	
25	B	1,000	3	78	
25	A	3,000	5†	77	
26	A	......	5†	76	
27	B	......	3	75	
3/ 2	B	5,400	2	73	
2	A	9,800	2	74	
3	A	35,000	1	76	Pool closed until 3/16. Filters continued in operation
16	B	900	0	74	
18	B	1,300	0	74	
18	A	2,400	2	73	
19	A	1,500	3	74	
20	A	1,700	0	74	
23	A	1,200	2	76	
31	B	2,500	1	76	Water low
4/ 3	B	1,200	0	76	Water low
3	A	8,000	0	76	Water low
4	B	700	0	74	Water low
6	B	1,250	0	72	Water low
7	A	440	0	72	18 inches of fresh water added
10	B	310	0	67	
10	A	1,850	0	70	
13	B	1,300	0	76	
13	A	1,800	1	77	
15	A	3,000	0	80	
16	A	8,400	1	78	
17	A	8,200	3	77	Pool emptied

* A1 = sample taken during use or immediately after.
  B2 = sample taken before pool was opened for the day.
† All **five** 1-c.c. samples tested were positive. There were therefore 5 or more gas-formers in 5 c.c.
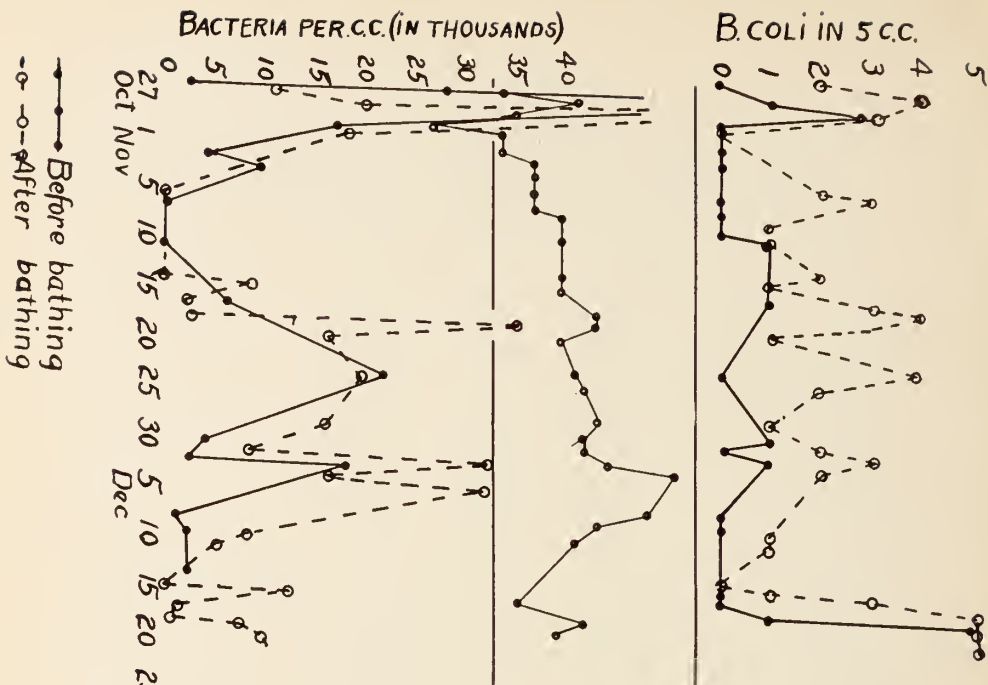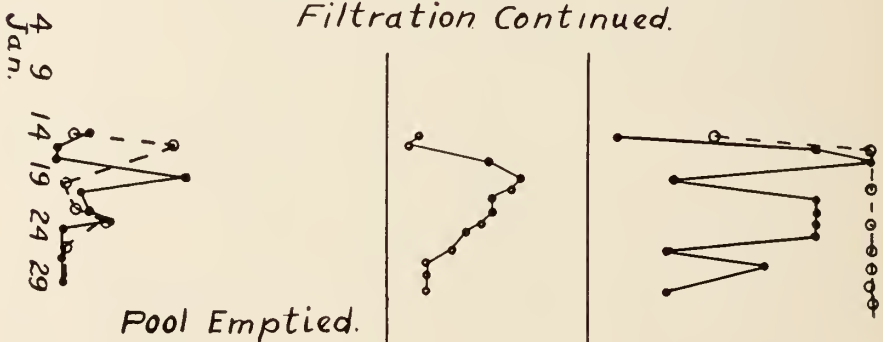
Chart 1. The results of the analyses of all samples from October 27, when the tank was put into operation, to February 1, when it was emptied and cleaned.

tion is that of direct contact with infectious material introduced by the bathers. Steps should be taken to make such material innocuous the moment it finds its way into the water. For this purpose, some disinfectant which is continuously acting and which is without effect on the bathers would be ideal. Copper sulfate, free chlorin, and bleach have been suggested. The last named, because of its success in water

TABLE 4

Bacterial Analyses of the Water in the Swimming Pool when Continuous Filtration was Supplemented with Periodic Disinfection with Calcium Hypochlorite

Date	Time	Bacteria per c.c. (Agar 37 C.)	B. coli in 5 c.c.	Remarks
1914 4/27	5:30 p. m.	17,200	3	0.5 part per million available chlorin as hypochlorite* added at 5:45, being passed up and down in cheese cloth
27	7 p. m.	154	0	
28	5:30	4,700	3	
30	5:30	......	2	
5/ 1	5:30	42,500	1	
4	5:30	3,500	5	0.63 part per million available chlorin as hypochlorite added in suspension at 6 p. m.
4	7:00 p. m.	270	2	
6	5:30 p. m.	165,000	5	
7	5:30 p. m.	235,000	5	
8	10:15 a. m.	90,000	5	0.44 part per million available chlorin added as hypochlorite in suspension at 10:15. Pool very muddy
8	11:15 a. m.	27,000	2	
9	6:00 p. m.	9,600		
1915 4/ 5	8 a. m.	700	0	
6	8 a. m.	4,000	0	0.75 part per million available chlorin as hypochlorite in suspension added at 6 p. m.
7	8 a. m.	28	0	
8	8 a. m.	12,000	0	
9	8 a. m.	52,000	0	
12	8 a. m.	34,300	0	
13	8 a. m.	7,600	0	0.75 part per million available chlorin as hypochlorite in suspension added at 6 p. m.
14	8 a. m.	81	0	
16	8 a. m.	9,100	2	
27	8 a. m.	2,500	0	0.75 part per million available chlorin as hypochlorite in suspension added at 6 p. m.
28	8 a. m.	17	0	
29	8 a. m.	1,400	0	

* Bleach contained 35.8% available chlorin (tested by J. S. Coye of the Engineering Experiment Station).

purification, has been highly recommended and most commonly employed. Tully,[7] Ravenel,[6] Lewis,[2] Manheimer,[1] Norton,[8] Rettger and Markley,[11] Bunker,[12] Burrage,[3] and others, have all recommended calcium hypochlorite and they suggest the addition of 0.5 to 1 part per million available chlorin 2 or 3 times a week.

[11] Eng. News, 1911, 66, p. 636.
[12] Am. Jour. Pub. Hyg., 1910, 6, p. 810.

Manheimer,[1] recognizing the desirability of continuous disinfection, advises the constant application of bleach in small quantities to the water as it passes into the filters. He states that this gradual addition is preferable to periodic applications of larger quantities to the tank proper. Clark and Gage[5] in a test of the swimming pool at Andover, Mass., found that the continuous addition of 0.3 part per million available chlorin to the water passing through the filters resulted in a purification of only 68%. They obtained more satisfactory results by the periodic application of bleach directly to the tank.

Results obtained with hypochlorite at Ames.—In the first of the experiments to be described, the disinfectant was placed in a bag and drawn across the surface. In all other tests the bleaching powder was suspended in water and sprinkled over the surface.

Table 4 shows that the application of from 0.5 to 0.75 part per million available chlorin generally brings about a reduction of over 98% in a few hours; but that the germicidal action is quickly lost, is indicated by the rapidly rising counts 24 to 48 hours after the introduction of hypochlorite. Available chlorin, 0.75 part per million, was much more efficient towards reducing the count of B. coli than towards reducing the total count.

Thomas,[10] in a study at Lehigh University, using 0.83 part per million available chlorin obtained similar results, from which he concludes: "To add 2.5 parts per million of the hypochlorite every day would probably solve the problem from a bacteriological standpoint, but the odor would be too offensive for this form of treatment to be considered."

Norton,[8] in a recent study of the Y. M. C. A. pool at Cambridge, Mass., employed 0.75 part per million available chlorin, or 1 pound hypochlorite in 55,000 gallons of water per day. The chemical was added every night after the bathers left. He concludes: "Addition of hypochlorite causes an immediate reduction in the number of bacteria, and this number is kept down by this and the rest of the purifying system to a reasonable limit for about four weeks. The same is true of the gas-forming elements. In the opinion of the author [Norton] the two main factors in keeping the bacterial count low are the addition of hypochlorite and the sedimentation . . ." The data he presents show that 75% of the samples had a bacterial content of from 18,000 to 300,000 on litmus lactose agar and that B. coli was present in numbers of from 1 to 10 in 50% of 5-c.c. samples. Unless we accept counts of from 18,000 to 300,000 as low for swimming pools, his conclusions as to the efficacy of hypochlorite are not warranted by the published data.

The work of Clark and Gage[5] on the value of hypochlorite in swimming pools is probably the most extensive yet reported. They found that daily application kept the count reasonably low for the first few weeks, but that soon the total and B. coli counts began to rise rapidly, necessitating an

increase in the amount of disinfectant. Their experience with the periodic use of bleach was similar. The count was low only when free chlorin was present in the water. Even with such enormous quantities as 3.7 parts per million available chlorin the count was kept down only for a day or two, and on days when an excess of hypochlorite was used, the number of bacteria exceeded 10,000 per cubic centimeter more than one-third of the time.

They draw the following conclusion: "The results . . . show quite clearly that occasional disinfection or regular disinfection [with hypochlorite] at intervals of several days cannot be relied upon to maintain a water of low bacterial content . . ." They also note that there were many complaints of burning and smarting eyes.

Enough has probably been said to point out that the use of bleaching powder has not solved the question of the sanitary control of swimming pools. The advocates of hypochlorite base their opinions largely on a priori reasoning from its success in water-purification. A swimming pool is not a water supply, except in the sense that the novice will use it as such. A water supply after treatment is protected against repollution. A swimming pool is constantly receiving pollution and should therefore be constantly disinfected. Roberts[13] points out that disinfection should be continuous, but that an occasional complete sterilization would be advantageous. Hypochlorite may be employed for an occasional sterilization, but its rapid decomposition, its irritating effect on the eyes, and possibly harmful influence on the teeth, eliminate bleach for use in continuous disinfection of swimming pools.

EFFECT OF CONTINUOUS FILTRATION AND DISINFECTION WITH COPPER SULFATE

That copper sulfate under proper conditions is an efficient germicide is well known, but its value as a swimming-tank disinfectant has not been adequately tested. Stokes[14] used $CuSO_4$ in 1 : 100,000 dilution and found that algae and bacteria were destroyed. Manheimer[1] found that of 6 pools practicing disinfection, 3 used $CuSO_4$. Rettger[11] had to abandon $CuSO_4$ on account of the hardness of the water, while Thomas[10] points out that with a hard water softened by treatment with alum and mechanical filtration, 0.4 part per million $CuSO_4$ was more efficient than 0.83 part per million hypochlorite.

During the summer of 1915, $CuSO_4$ was substituted for bleach at the Iowa State College and the results observed for 3 weeks. The bacteriologic analyses are given in Table 5. During the observation period 4 pounds of the chemical were added. The disinfectant was

[13] Eng. News, 1912, 67, p. 73.
[14] Am. Med., 1905, 10, p. 1075.

TABLE 5

BACTERIAL ANALYSIS OF WATER IN THE POOL AT IOWA STATE COLLEGE WITH CuSo₄ DISINFECTION AND CONTINUOUS FILTRATION

Date	Time*	Bacteria per c.c. † (Agar 37 C.)	B. coli in 5 c.c.	Remarks
1915				
7/ 5	B	80	5	Before addition of CuSo₄
5	A	9	0	1½ hr. after adding 1.5 part per million of CuSo₄. Pool used by 70 boys. Water in pool muddy
6	B	36	0	
7	B	19,300	0	
8	B	1,870	0	Pool cleaned. 1.5 parts per million CuSO₄ added after taking sample
8	A	178	0	3 hr. after addition of CuSo₄. Pool used by 80 boys
9	B	155	0	
10	B	142	0	
13	B	57	0	3 parts per million CuSo₄ added after taking sample
13	A	4	0	3 hr. after adding CuSo₄. Pool used by 70 boys
14	B	11	0	
15	B	12	0	
16	B	50	0	
19	B	120	0	18 inches of fresh water added
20	B	535	0	
21	B	2,520	0	
22	B	1,900	0‡	

* B = before pool was opened for use.
A = after pool was used, usually 2 hours.
† These counts were made on agar in which the peptone of the Digestive Ferments Co. was used, while previous counts were made with Witte's peptone. The bile for the presumptive tests for B. coli was made with Witte's peptone in all cases.
‡ B. coli present in 10 c.c.

TABLE 6

BACTERIAL CONTENT OF THE IOWA STATE COLLEGE SWIMMING TANK WITH DIFFERENT METHODS OF TREATMENT

Bacteria per c.c. (Agar 37 C.)	Percentage of Samples		
	Continuous Filtration	Filtration and Disinfection with Calcium Hypochlorite	Filtration and Disinfection with Copper Sulfate
0-100	5	15	47.
101-500	15	10	23.5
501-1000	7	0	5.9
1001-5000	34	25	17.7
5001-10,000	14	15	5.9
10,001-50,000	21	15	0
50,001-100,000	4	10	0
Over 100,000	0	10	0
B. coli in 5 c.c.			
Less than 1	29.5	52.5	94.1
1	19.0	9.5	0
2	11.5	19.0	0
3	11.4	0	0
4	8.6	0	0
5 or more	20.0	19.0	5.9

introduced just before the pool was opened. A bag containing the $CuSO_4$ was drawn along the surface of the water until all was dissolved. This procedure usually took about 15 minutes.

From Table 5 it is apparent that, under the conditions present in this pool, $CuSO_4$ is very efficient in keeping down the total bacterial count, and also the count of B. coli.

A comparison of the results obtained with filtration alone, with filtration supplemented by disinfection with hypochlorite, and with filtration supplemented by copper sulfate, leads to the conclusion that the latter method is the most efficient. This is shown in Table 6.

B. coli was absent from 29.5% of 5-c.c. samples before disinfectants were employed; from 52.5% of samples after hypochlorite was used; and from 94% after copper sulfate. The total bacterial counts particularly, indicate that copper sulfate is superior to bleach as a disinfectant for swimming pools. If we compare copper sulfate and bleach when the latter was employed in quantities of 0.75 part per million, or more, available chlorin, we shall find that they had about the same effect on B. coli—94% of 5-c.c. samples were negative with the copper sulfate and 91% with bleach; but the total bacterial content was considerably lower with copper sulfate (see Tables 4 and 5).

SUMMARY AND CONCLUSIONS

Swimming pools are being rapidly adopted throughout the country. Unless they are adequately protected against infectious matter and their sanitary conditions controlled, they may serve as a vehicle for the transmission of disease.

A sanitary standard for plunges would be desirable, but it is necessary first to standardize the methods of analysis in order to obtain comparable data on which to base such a standard.

At the pool of the Iowa State College continuous filtration effected a reduction of 60% in the bacterial count. For 6 weeks presumptive tests for B. coli in 5 c.c. before use of the pool, were usually negative. After that time positive tests became more frequent until gasformers were constantly present in 1 c.c. and occasionally in 0.1 c.c. During this period the total count on agar at 37 C. decreased irregularly.

With continuous filtration and without disinfection the sanitary condition of the pool, as indicated by the total and the B. coli counts,

was better when the temperature of the water was below 74 F. than it was when above. The temperature of the room should be higher than that of the water.

Continuous filtration alone is insufficient to maintain a tank in a sanitary condition. Filtration should be supplemented with disinfection.

The bathers are constantly introducing pollution and possibly infectious matter. To off-set this continuous pollution disinfection must also be continuous and the disinfectant should be most effective at the time when the pool is in use. It is in these respects that the periodic application of such substances as calcium hypochlorite, after the tank is vacated, falls down. Bleach is excellent for an occasional or terminal sterilization, but on account of its irritating effect on the eyes and possibly harmful influence on the teeth, it cannot be employed efficiently for continuous disinfection.

Copper sulfate is very efficient for swimming-pool disinfection. It is cheaper, has no irritating effect, its disinfecting action is slower, and consequently more applicable to continuous disinfection, than that of hypochlorite. The copper sulfate could be added just before the pool is opened and thus its maximal efficiency would be exerted during the period of maximal pollution. Filtration supplemented by disinfection with 1 part per million $CuSO_4$ 3 times a week and, if desired, an occasional sterilization with calcium hypochlorite will keep a swimming tank in good sanitary condition for several months.

EXPERIMENTAL CHOLERA-CARRIERS *

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The following series of experiments was undertaken to determine definitely whether animals could be made carriers of the cholera vibrios, the length of time they would remain carriers, and the nature of the condition, the object being to subject such experimental carriers to various methods of treatment, some of which have been recommended in practice, while others are based on theory.

Technic

Inoculation.—The culture used, isolated during the recent outbreak of cholera in Austria, was furnished by Karl Landsteiner of Vienna, and was known at the laboratory as Cholera Austria 4. The culture was inoculated into ox bile and transplanted daily for a week before the experiments were begun, as cholera vibrios had been observed to remain alive in bile for a considerable length of time, in lively motility.

In the inoculation of the gall-bladder, the stomach, and the intestine, rigid antiseptic measures were adopted to avoid accidental infection. The animals were shaved, the skin washed with lysol solution, and then painted with tincture of iodin. For an inoculation of the stomach or of the small intestine, the abdominal cavity was opened in the middle line in the epigastric region.

The gall-bladder in guinea-pigs is rather free and of comparatively large size. It is located between the lobes of the liver (only a small part of it being attached to the liver), in the angle formed by the right costal margin and the xiphoid process. In performing the operation an incision was made from the middle of the xiphoid process to the right costal margin. As soon as the muscles were separated, the peritoneum became visible, and through it the xiphoid process. The latter was clasped with a hemostat and lifted up, whereupon the duplicature of the peritoneum formed thereby was perforated by means of a dull forceps. As a rule, the gall bladder was immediately visible and, prolapsing into the laparotomy-wound, it closed the opening, thus preventing exposure of the other organs. The injection could be made therefore outside the peritoneal cavity. Only fractions of a cubic centimeter were injected into the gall-bladder. The inoculation finished, the puncture in the gall-bladder was closed with a ligature, the ends of the wound were lifted up, and the gall-bladder assumed its normal position. Silk threads thoroughly soaked with tincture of iodin, were used for suturing the abdominal wound.

Intravenous inoculation was made in the usual way.

For inoculation by feeding the animal was held on a tray in frog position with head up. The outlet of a graduated pipet containing the bacterial suspension was placed in its mouth and the suspension allowed to flow into the pharynx. Tame animals, if handled cautiously, will take several cubic centi-

meters of liquid without spilling a drop. The feeding finished, the mouth of the animal was wiped off with a piece of cotton soaked in lysol and the tray thoroughly disinfected.

Inoculated animals were kept either in glass jars or in solid galvanized-iron cages. These were disinfected at regular intervals. The feeding of the animals, and the cleaning and disinfection of the containers, were attended to by the author.

Examination.—To ascertain whether or not the animals became infested with cholera vibrios they were killed and examined immediately. The shaved skin over the chest and abdomen was wetted with 2% lysol solution. The abdomen and thorax were opened separately. The gall-bladder including the bile duct, the stomach, the proximal part of the intestine (in the reports, duodenum), the distal part of the small intestine (in the reports, the ileum), the cecum, and the distal part of the large intestine (in the reports, rectum) were double ligated, and then removed from the abdomen—first the gall-bladder, then the duodenum, ileum, stomach, cecum, and rectum, in order, a separate set of sterilized instruments being used for each organ. The gall-bladder was taken out in toto, including a portion of the bile duct. The contents of the gall-bladder were emptied into peptone water, and the gall-bladder together with the bile duct placed in a culture tube. The contents of the proximal part of the small gut were planted, and the intestine, cut into small pieces, was added to the same peptone tube. At least one-half of the distal part of the small intestine was planted in the same way as the duodenum. The stomach was opened on the large curvature, and 5 large loopfuls of the contents inoculated into peptone water. The same amount of the contents of the cecum was planted in a similar way. One portion of formed feces obtained from the distal part of the large intestine, was crushed with sterile forceps and placed in peptone water. During life, shortly after feeding, the animals were placed in thoroughly disinfected glass jars, and the feces collected as soon as deposited, peptone cultures being made in the same way as that described.

The peptone cultures thus obtained were sub-planted, after from 6 to 8 hours' incubation, into another set of tubes containing peptone water. The first and second peptone cultures were examined microscopically from time to time and plated on Dieudonné medium after 12 and 18 hours, respectively.

INTRAVESICULAR INOCULATION

Four guinea-pigs were inoculated in the gall-bladder. An alkaline-agar subculture was used, 0.1 of one slant being injected.

Guinea-pig 1.—Killed and examined 24 hours after inoculation. Peptone cultures from gall-bladder, ileum, and cecum were positive for cholera vibrios.

Guinea-pig 2.—Killed and examined 2 days after inoculation. Peptone cultures from gall-bladder, duodenum, and ileum were positive; the culture from the cecum was negative.

Guinea-pig 3.—Killed and examined 4 days after inoculation. Peptone cultures from gall-bladder, duodenum, ileum, and cecum were positive.

Guinea-pig 4.—Killed and examined 7 days after inoculation. Peptone cultures from the gall-bladder, duodenum, ileum, and cecum were positive; those from stomach and rectum were negative.

Four guinea-pigs inoculated in the gall-bladder harbored cholera vibrios. The animals were examined 1, 2, 4, and 7 days after inoculation.

INOCULATION OF THE STOMACH

Five guinea-pigs were inoculated in the stomach. Magnesium oxid suspended in water was given by mouth previous to the operation. Cholera Austria 4 was used, 0.025 of a slant being injected. The culture had been transplanted daily in ox bile for a week, passed through one rabbit, and recovered from the gall-bladder of the animal 13 days after inoculation.

Guinea-pig 5.—Examined 2 days after inoculation. It had died of pneumonia. Peptone cultures from gall-bladder, stomach, duodenum, ileum, and rectum were positive.

Guinea-pig 6.—Examined 3 days after inoculation. Peptone cultures from gall-bladder, duodenum, ileum, and cecum were positive.

Guinea-pig 7.—Examined 4 days after inoculation. It had died of pneumonia. Peptone cultures from gall-bladder, stomach, duodenum, and ileum were positive; those from cecum and rectum were negative.

Guinea-pig 8.—Examined 7 days after inoculation. Peptone cultures from gall-bladder, stomach, duodenum, ileum, and cecum were negative.

Guinea-pig 9.—Examined 8 days after inoculation. Peptone cultures from gall-bladder, duodenum, and ileum were positive; those from stomach, cecum, and rectum were negative.

Four of the 5 guinea-pigs inoculated, harbored cholera vibrios 2, 3, 4, and 8 days after inoculation. One animal of 5 was negative 7 days after inoculation.

INOCULATIONS OF THE SMALL INTESTINE

Five guinea-pigs were inoculated in the small intestine after the same method as in the case of the stomach.

Guinea-pig 10.—Examined 2 days after inoculation. Peptone cultures from gall-bladder, duodenum, ileum, and cecum were positive.

Guinea-pig 11.—Examined 7 days after inoculation. Peptone cultures from gall-bladder, duodenum, ileum, and cecum were negative.

Guinea-pigs 12 and 13.—Examined 8 days after inoculation. Peptone cultures from gall-bladder, stomach, duodenum, ileum, and cecum were negative.

Guinea-pig 14.—Examined 10 days after inoculation. Peptone cultures from gall-bladder, duodenum, ileum, stomach, and cecum were negative.

One of the 5 animals inoculated in this manner harbored cholera vibrios 2 days after inoculation. Four of the 5 guinea-pigs, examined 7, 8, and 10 days after inoculation, were negative.

INOCULATION BY FEEDING

Five guinea-pigs were inoculated by feeding. The culture, Cholera Austria 4, had been transplanted daily in ox bile for a week and passed through one guinea-pig. One tenth of a slant was fed, magnesium oxid being given with the culture.

GUINEA-PIGS 15, 16, AND 17.—Examined 3 days after inoculation. Peptone cultures from gall-bladder, stomach, duodenum, ileum, and cecum were negative.

GUINEA-PIGS 18 AND 19.—Examined 4 days after inoculation. Peptone cultures from gall-bladder, stomach, duodenum, ileum, and cecum were negative.

Not one of the 5 animals, examined 3 and 4 days after inoculation, harbored cholera vibrios.

Three guinea-pigs, unfed for 18 hours, were given magnesium oxid by mouth. One and a half hours later they were fed 2 c.c. of culture, Cholera Austria 4, grown in ox bile for 24 hours. (The culture had been transplanted daily in ox bile and passed through one guinea-pig.) All the animals became sick, took little food, and had diarrhea.

GUINEA-PIG 20.—Examined 20 hours after inoculation. Intestine distended; contained bile. Gall-bladder about twice the normal size. Peptone cultures from gall-bladder were negative; those from stomach, duodenum, ileum, and cecum were positive.

GUINEA-PIG 21.—Examined 3 days after inoculation. The gall-bladder was distended to twice the normal size. Peptone cultures from duodenum, ileum, and cecum were positive; those from gall-bladder and rectum were negative.

GUINEA-PIG 22.—Examined 7 days after inoculation. The gall-bladder was of normal size. Peptone cultures from gall-bladder, stomach, duodenum, ileum, and cecum were negative.

This experiment was repeated, each guinea-pig receiving one slant of agar culture suspended in alkaline peptone solution. The culture had been transplanted daily in ox bile, passed through one guinea-pig, and recovered from the gall-bladder of the animal 4 days after inoculation.

GUINEA-PIG 23.—Examined 20 hours after inoculation. Peptone cultures from ileum and cecum were positive; those from gall-bladder, stomach, and duodenum were negative.

GUINEA-PIG 24.—Examined 3 days after inoculation. Peptone cultures from gall-bladder, stomach, duodenum, ileum, and cecum were negative.

GUINEA-PIG 25.—Examined 7 days after inoculation. Peptone cultures from gall-bladder, duodenum, and ileum were positive; those from stomach, cecum, and feces were negative.

The experiment was again repeated. The culture had been transplanted daily in ox bile for a week, passed through 2 guinea-pigs, and recovered from the gall-bladders of the animals 4 and 7 days after

inoculation. In the experiment each animal received one agar slant suspended in alkaline peptone water.

Guinea-pig 26.—Examined 3 days after inoculation. The peptone culture from the ileum was positive; cultures from stomach, gall-bladder, duodenum, cecum, and feces were negative.

Guinea-pig 27.—Examined 8 days after inoculation. Peptone cultures from gall-bladder, stomach, duodenum, ileum, cecum, and feces were negative.

Guinea-pig 28.—Examined 14 days after inoculation. Peptone cultures from gall-bladder and ileum were positive; those from stomach, duodenum, cecum, and feces were negative.

From the last 3 experiments it is seen that the gall-bladder in animals which were killed and examined early after inoculation by feeding was free from cholera vibrios. Those animals which were killed and examined at later periods after inoculation, if they harbored cholera vibrios at all, showed them present in the gall-bladder.

INOCULATION BY INTRAVENOUS INJECTION

Two guinea-pigs were inoculated by intravenous injection. The culture, Cholera Austria 4, had been transplanted daily in ox bile, passed through 2 guinea-pigs, and recovered from the gall-bladders of the animals 4 and 17 days after inoculation. In the experiment a minute amount of cholera culture was injected. The animals were sick after the injection, but recuperated in 24 hours.

Guinea-pig 29.—Examined 4 days after injection. Peptone cultures from gall-bladder, stomach, duodenum, ileum, cecum, and feces were negative.

Guinea-pig 30.—Examined 12 days after inoculation. Peptone cultures from stomach, gall-bladder, duodenum, ileum, cecum, and feces were negative.

Four guinea-pigs (64, 65, 66 and 67) were inoculated by intravenous injection of 0.1 of a slant, Cholera Austria 4 being used. (The culture had been transplanted daily in ox bile for a week, passed through 6 guinea-pigs, and recovered from the gall-bladders of the animals, 7, 17, 6, 13, 1, and 5 days after inoculation.) The animals became sick after the injection and were found dead the next morning. Peptone cultures made from heart blood, spleen, lung, liver, gall-bladder, ileum, and cecum were positive for cholera vibrios.

An attempt to infest guinea-pigs by intravenous injection of small and large amounts of living cholera vibrios failed.

INOCULATION BY INJECTION OF CULTURE INTO THE SEROUS CAVITY

Three guinea-pigs were inoculated by injection of the culture into the right pleural cavity, Cholera Austria 4, 0.1 of one slant, being used.

The culture had been transplanted in ox bile daily for a week, passed through 6 guinea-pigs, and recovered from the gall-bladders of the animals 7, 17, 6, 13, 1, and 5 days after inoculation.

GUINEA-PIG 71.—Killed in ultimis and examined 24 hours after inoculation. Peptone cultures from pleura, peritoneum, blood, lungs, spleen, and liver were positive.

GUINEA-PIG 72.—Examined 2 days after inoculation. Peptone cultures from pleura, peritoneum, blood, lungs, spleen, liver, gall-bladder, duodenum, ileum, and cecum were negative.

GUINEA-PIG 73.—Examined 5 days after inoculation. Peptone cultures from pleura, peritoneum, blood, lungs, spleen, liver, gall-bladder, duodenum, ileum, and cecum were negative.

Of all the modes of inoculation the intravesicular injection showed the highest percentage of "takes." This method was adopted in the further course of the investigation.

TABLE 1

THE DISTRIBUTION OF CHOLERA VIBRIOS IN THE ALIMENTARY SYSTEM OF GUINEA-PIGS AND THE DURATION OF THE CARRIER STATE AFTER INTRAVESICULAR INOCULATION

Guinea-pig	Days After Inoculation	Gall-bladder	Stomach	Duodenum	Ileum	Cecum	Rectum
1	1	+	0	0	+	+	0
2	2	+	0	0	+	+	0
62	3	+	—	+	+	+	0
68	3	+	—	+	+	+	0
69	3	+	+	+	+	+	+
3	4	+	0	+	+	+	0
56	4	+	—	+	+	+	+
65	4	+	0	+	+	+	—
49	5	+	—	+	+	+	—
50	5	+	—	+	+	+	—
63	5	+	—	+	+	+	—
34	6	+	—	+	+	—	—
40	6	+	0	+	+	—	—
41	6	+	0	+	+	+	—
54	6	+	—	+	+	—	—
55	6	+	—	+	+	+	—
57	6	+	—	+	+	+	—
58	6	+	—	+	+	—	—
4	7	+	—	+	+	+	—
35	7	+	—	+	+	+	—
59	7	+	—	+	+	—	—
66	8	+	—	+	+	+	—
67	8	+	—	+	+	+	—
70	8	+	—	+	+	+	—
32	10	+	—	+	+	+	—
60	10	+	—	+	+	+	—
33	11	+	—	+	+	+	—
46	13	+	—	—	+	+	—
47	13	+	—	+	+	+	—
48	13	+	—	+	+	+	—
64	16	—	—	—	—	—	—
36	17	+	—	+	+	+	—
43	30	—	—	—	—	—	—

+ = cholera vibrios present in the culture.
— = cholera vibrios absent in the culture.
0 = not examined.

The results of the examinations made on 33 experimental cholera-carriers are summarized in Table 1, which shows also the percentage

of "takes," the distribution of cholera vibrios throughout the various parts of the alimentary canal, and the duration of the condition in question.

The excretion of cholera vibrios in the feces of the animals deserves special mention. It will be seen from the examples of examination of feces for the presence of cholera vibrios that the successfully infested animals do excrete cholera vibrios in their feces. It is probably due to the peculiar structure of the gut and to the slight resistance of the cholera vibrio to unfavorable conditions that it does not occur as regularly as one would expect. Guinea-pigs 74, 76, and 77 serve as examples of the fact, already known with regard to human beings, that cholera vibrios can pass through the entire alimentary canal without getting a foothold in the body.

EXAMPLES OF EXAMINATION OF FECES FOR THE PRESENCE OF CHOLERA VIBRIOS
DURING THE LIFE OF EXPERIMENTAL GUINEA-PIG CHOLERA-CARRIERS

43.—Intravesicular inoculation. On the 8th, 9th, 10th, 11th, and 12th days after inoculation, negative; on 13th day, positive; on 15th, 16th, 17th, 18th, and 20th days, negative.

43a.—Intravesicular inoculation. On the 1st day, negative; on the 2nd, 3rd, and 4th days, positive.

44.—Intravesicular inoculation. On the 1st day, negative; on the 2nd, 3rd, and 4th days, positive.

45.—Intravesicular inoculation. On the 1st, 2nd, 3rd, 4th, 6th, 9th, 10th, 12th, and 13th days, negative.

46.—Intravesicular inoculation. On the 1st, 2nd, 5th, 8th, and 9th days, negative; on the 10th day, positive; on the 12th day, negative.

47.—Intravesicular inoculation. On the 1st, 2nd, 5th, 7th, 8th, 9th, and 10th days, negative; on the 12th day, positive.

48.—Intravesicular inoculation. On the 1st, 2nd, 5th, 6th, 8th, and 10th days, negative; on 12th day, positive.

49.—Intravesicular inoculation. On the 1st, 2nd, and 5th days, negative.

50.—Intravesicular inoculation. On the 1st day, negative; on the second day, positive; on the 5th day, negative.

60.—Intravesicular inoculation. On the 3rd day, positive; on the 4th day, negative.

63.—Intravesicular inoculation. On the 4th day, negative.

64.—Intravesicular inoculation. On the 2nd, 4th, 6th, 8th, and 9th days, negative.

65.—Intravesicular inoculation. On the 2nd and 3rd days, negative.

66.—Intravesicular inoculation. On the 3rd day, positive.

67.—Intravesicular inoculation. On the 2nd day, positive.

68.—Intravesicular inoculation. On the 2nd day, negative.

69.—Intravesicular inoculation. On the 2nd day, positive.

70.—Intravesicular inoculation. On the 2nd day, negative.

74.—Inoculation by feeding. On the 1st day, positive; on the 2nd and 3rd days, negative. Animal killed. Gall-bladder, stomach, and intestines negative.

75.—Inoculation by feeding. On the 1st, 2nd, and 3rd days, negative. Animal killed. Gall-bladder, stomach, and intestines, negative.

76.—Inoculation by feeding. On the 1st day, positive; on the 2nd and 3rd days, negative. Animal killed. Gall-bladder, stomach, and intestines, negative.

77.—Inoculation by feeding. On the 1st day, positive; on the 2nd and 3rd days, negative. Animal killed. Gall-bladder, stomach, and intestines negative.

SUMMARY

Attempts were made to produce in animals a condition which would resemble that of cholera-carriers in human beings. Inoculations of cholera vibrios into the gall-bladder, stomach, small intestine, blood stream, and serous cavity were made and inoculation by feeding was also tried.

Direct inoculation into the gall-bladder, stomach, and small intestine and inoculation by feeding proved successful inasmuch as a certain percentage of the inoculated animals were found to harbor cholera vibrios in the alimentary canal. This was ascertained by bacteriologic examination of various parts of the digestive system, made in the great majority of cases immediately after death.

The intravesicular inoculation proved to be far superior to other methods. Practically every one of the animals inoculated in this way harbored cholera vibrios.

The duration of the condition, altho limited, appears to be sufficiently long for therapeutic experiments.

THE LOCALIZATION OF STREPTOCOCCI IN THE EYE [*]

A STUDY OF EXPERIMENTAL IRIDOCYCLITIS IN RABBITS

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The tendency of organisms to invade special tissues of the body is one of the fundamental facts in the etiology and pathology of disease. In the case of some organisms this tendency is the rule, so that the meningococcus usually localizes in meninges, the pneumococcus in the lung, the gonococcus in joints and tendon sheaths, tho at the onset each is present in the blood stream.

Other common pathogenic bacteria, such as the streptococcus, show a more diverse and less constant localization, and while there are in some instances distinguishable differences in cultural characteristics between types and races of streptococci producing different lesions, in many cases streptococci isolated from various sites, and giving rise to varying clinical pictures, show no such cultural or morphologic differences. It has been repeatedly pointed out that the peculiarities of various organs and tissues as regards blood supply, trauma of use, and the special requirements of the invading organism with respect to protection from unfavorable influences, food supply, and oxygen tension, probably have much to do with determining the localization of subsequent generations of the organism.

Organisms long resident in some focus in the body, such as the tonsils, may spontaneously and suddenly invade other tissues, and set up new processes, which present clinical pictures entirely different from those produced before; organisms from these new lesions may cause similar lesions in animals. Rosenow found that streptococci isolated from the tonsils and from the wall of the appendix in patients suffering from appendicitis produced appendicitis in rabbits; that streptococci from patients suffering from arthritis produced a large proportion of joint lesions; and that streptococci from cholecystitis produced cholecystitis in animals after intravenous injection. As a rule, these peculiar qualities of localization were lost early, sometimes within a very few days after isolation. Occasionally during a series of animal passages, the tendency of an organism to localize in one organ or type of tissue changed so that localizations became more frequent in other organs.

Streptococcal infections of extremities, such as a subungual infection of finger or toe, have been observed to give rise to a general invasion of the body, with arthritis conforming in clinical type not to that of an ordinary streptococcal sepsis, but to that of a typical attack of acute rheumatic fever.

Of the finer mechanism by which organisms acquire for brief periods this invasive power for special organs or tissues we know little, but the recognition that organisms may take on such new qualities offers an attractive explanation of many hitherto obscure phenomena in disease.

The brief duration of this new localization, frequently the relative insignificance in point of size and clinical manifestations of the original focus which harbored the invader, and the relatively slight systemic disturbance accompanying the event until the development of symptoms in the organs newly invaded, have obscured the relation between the disease and its original focus.

The metastatic origin of lesions of the eye, such as panophthalmitis in streptococcal or pneumococcal sepsis, has long been clearly recognized, and the metastatic origin of similar or less severe lesions of the eye in patients in whom the other symptoms of bacterial invasion are slight or absent, or confined to some focus elsewhere in the body, is now being recognized in an increasing proportion of cases. The argument for the bacterial origin of such lesions of the eye is strengthened by the occurrence in some patients of a preceding or coincident arthritis, in the course of which the presence of bacteria can be demonstrated in the joints, and sometimes in the blood. Stock and others have produced iridocyclitis in animals by intravenous injections of bacteria. Recently Rosenow[1] has noted iridocyclitis in a number of animals, chiefly rabbits, following the intravenous injection of streptococci and other organisms derived from various lesions in man and in animals.

While we believe with Rosenow that the localization of organisms (streptococci) in the eyes (uveal tract) of rabbits in his experiments probably was due in large part to peculiarities of the organisms themselves, we recognize the justice of the argument of those who would maintain that on the theory of probabilities, bacterial embolism of vessels of the eye may be expected in a certain proportion of animals in which large amounts of organisms are introduced by intravenous injection. It might further be urged in support of this accident theory that

[1] Jour. Infect. Dis., 1915, 17, p. 403.

in the same animals in which lesions of the eye occurred, Rosenow found lesions of other organs (of 48 animals showing eye lesions, 28 showed arthritis, 5 hemorrhages in the appendix, 8 ulcer of the stomach, 10 lesions of the endocardium, 8 of the pericardium, 19 of the muscles and the fascia, and 12 of the kidneys), indicating a widespread bacterial invasion. Furthermore, these animals were individuals selected from several hundred similarly inoculated animals.

If, however, as Rosenow points out, the sources of the cultures used for these injections are considered, it appears that lesions of the eye were often observed in animals which received cultures of organisms derived from patients suffering from arthritis, myositis, etc., and rarely in animals which were injected with cultures from appendicitis, ulcer of the stomach, or cholecystitis. The source of the cultures therefore seems to have been an important factor in determining the percentage of lesions of the eyes following intravenous injection.

The following experiments were made with the idea of tracing the changes in the power of an organism (streptococcus) which had already given rise to iridocyclitis in a patient, to produce similar lesions in animals after varying periods of residence in the original host, of residence in animal tissues, and of growth on culture media. The accessibility of lesions of the eye to observation greatly facilitates studies of this sort. The exact time of the appearance of the lesions and their course can be more readily noted than when the lesions are in joints or other organs. Minor lesions which might escape notice, or disappear before the death of the animal when they involve joints or internal organs, are more easily detected in the eye.

The patient, J. S., aged 40, a waiter, single, white, was admitted to the Cook County Hospital, April 19, 1915. Eight days before, the patient had "caught cold" and his left tear sac had become swollen. Three days before, it had been opened at a neighboring dispensary. There had been pain in the left side of the head from onset of the eye trouble.

Following an operation upon his nose 20 years before, for the relief of some suppurative process involving the septum, the left eye had "watered" for years, and he had thenceforth been able to "press pus" out of the corner of his eye for 2 or 3 days at a time, at intervals of 5 or 6 months. The sac region had never been swollen and tender until 8 days before admission. About 12 days before admission, an eruption had appeared in an irregular band in the region of the right 9th and 10th ribs (herpes zoster). This had not been accompanied by any marked systemic disturbance. Aside from these lesions, and measles, whooping cough, and mumps in childhood, the patient had suffered no severe illnesses. He had had gonorrhea 12 years before, which had healed without observed complications. He denied syphilitic infection. One brother had died of tuberculosis; 4 sisters and 1 brother were living and well.

The patient was a moderate smoker of tobacco, and took alcoholics freely at times. His general health was good, appetite fair, bowels constipated on admission, urination free.

He was well-developed. Scalp, skull, and ears, appeared normal. The nose showed an old scar over the bridge; the septum was short and the end of the nose depressed. The tongue was coated, the tonsils enlarged, showing some hyperemia. The teeth were carious; radiographs showed 3 alveolar abscesses. The affected roots were extracted (Dr. Puterbaugh). The sinuses on admission were normal clinically (Dr. Friedberg), and radiographs showed normal densities (Dr. Blaine). Later the left antrum filled with pus by direct extension from the tear sac following an operation.

A number of healing lesions of the skin extended in a band around the chest from midsternum to the spine on the right side at the level of the 8th to the 10th ribs (herpes zoster). Lungs, heart, abdominal viscera, and reflexes, appeared normal. The toes of the left foot were deformed (old injury). There were no other joint lesions. The prostate was moderately enlarged, of uniform consistence, and not hard or tender.

Throughout the course of illness there was little constitutional disturbance and the temperature did not rise above 99.2 F.

Wassermann and gonococcal complement-fixation tests were negative. The urine was normal. Tuberculin tests, made after subsidence of the iritis, were negative after 1 and 4 milligrams subcutaneously.

Blood cultures on April 28 (17 days after onset of iritis) and on May 28, made on plates, in tall dextrose-agar tubes, and in ascites dextrose broth, gave no growth.

Cultures from the tear sac, April 28, contained many streptococci and a few colonies of a white staphylococcus. The streptococcus grew in long chains, produced a wide zone of hemolysis, fermented dextrose, lactose, saccharose, and salicin, but not raffinose, mannite, or inulin. Cultures from the tear sac on 6 subsequent occasions gave approximately the same relative numbers of streptococci and staphylococci.

Smears from the tonsils showed streptococci with a smaller number of gram-negative diplococci. This streptococcus (S. viridans) produced small green non-hemolytic colonies on blood agar. Dextrose, saccharose, lactose, and raffinose were fermented, but not salicin, mannite, or inulin. Two rabbits injected intravenously with cultures direct from the tonsils, developed no lesions of the eyes. One rabbit showed a flaccid paralysis of the hind legs within 2 hours after injection and died the following day. There was a large infarct of the lung with multiple small infarcts and subpleural hemorrhages, 2 small subendocardial hemorrhages, and injection of the vessels of the pia with multiple hemorrhages almost continuous in the lower half of the cord. The second rabbit survived for 3 weeks without symptoms. No anatomic cause of death was found; a gram-positive bacillus was isolated from the heart blood.

The region of the left tear sac was much swollen, the skin over it red, tense, and tender. Large quantities of pus could be expressed. The left eye showed marked ciliary and conjunctival injection. A long narrow slightly raised epithelial vesicle ran along the entire margin of the upper inner corneal limbus; a shorter one lay just inside the lower outer limbus. The stroma beneath was distinctly furrowed out. Temporal to the upper furrow the marginal stroma was superficially vascularized for a width of 1 mm. along the limbus between 12 and 2:30 o'clock. The cornea was otherwise clear. The iris had a greenish discoloration (fellow iris, blue). The pupil dilated ad maximum up and in, less well upward and outward, and least well down and inward.

Posterior synechiae involving all layers of the iris were found at 8 and 5 o'clock, and the pupillary border was straight from 3 to 6 o'clock. There was no definite pupillary exudate, but the disc was seen hazily.

The conjunctiva of the right eye was somewhat injected, the pupil round, regular in outline, reacting to light and accommodation.

Three weeks after admission the acute swelling of the tear sac had subsided and it was fully opened and drained May 7 (Dr. Clark). No necrosis of bone was found. In the 5th week the tear sac was again opened and drainage into the nose effected. Some necrotic bone in the region of the ethmoid cells was found and curetted May 25. The center of the nasal quadrant of the cornea now showed a yellowish-white dot of infiltration, located in the anterior stroma and measuring only ⅓ mm. in diameter. In the course of the next few days 3 or 4 other areas appeared nearby. At first they were merely vesicles; then they broke and tiny areas of gray infiltration appeared beneath the pitted surface. The limbic vesicles also collapsed on the furrowed-out stroma and all gradually healed, very much as phlyctenulae do. Ciliary injection and conjunctival chemosis were marked. The pupil dilated well, but the iris remained greenish, considerably swollen, with markings blurred. Brown dots of detached iris pigment were found on the lens capsule.

In the 8th week the adjoining antrum, previously free of infection so far as could be determined clinically and by transillumination and radiographs, was found full of pus and connected directly with the tear sac. The antrum was drained through the nose. Bloody pus was obtained by lavage (Dr. Friedberg). In the 12th week the pus disappeared from both the tear sac and the antrum; the iritis was relieved and did not return in 10 weeks of subsequent observation.

THE PRODUCTION OF IRIDOCYCLITIS IN ANIMALS

Cultures taken from the tear sac of the patient, after varying periods of growth on laboratory media, were injected intravenously into rabbits. For convenience of discussion, the animals have been divided into 7 series, each series including the animals inoculated with cultures taken from the patient on the date indicated. The series therefore afford opportunity of studying changes in the invasive power of the streptococcus with respect to the eye and other organs of rabbits after varying periods of residence in the human body.

SERIES I

Four rabbits were injected intravenously with suspensions in salt solution of the 3rd and 4th subcultures on human-blood agar of a hemolytic streptococcus isolated from the tear sac of the patient on April 28. Iritis appeared in 3 of these rabbits (Rabbits 1, 3, 4).

Rabbit 1.—May 15, injected intravenously with a suspension from one 24-hour blood-agar slant (3rd subculture) of a hemolytic streptococcus from the tear sac of the patient.

May 19.—Right eye red, watery, with distinct conjunctivitis; vessels injected. Anterior chamber clouded. Dark iris, of darker color than normal left. Iris thrown into radial folds. Exudate adherent to anterior surface of iris, floating over pupil, appearing as tho squeezed from a narrow tube.

May 20.—Animal killed. No streptococci obtained in cultures from aqueous. Sections, however, disclosed many streptococci. Hemolytic streptococci in cultures from heart blood. No gross lesions of viscera.

Rabbit 3.—May 19, intravenously injected with a suspension of a culture from a 24-hour blood-agar-slant transfer from the culture used for Rabbit 1.

May 20.—Eyes unchanged.

May 22.—Severe inflammation in left eye, with conjunctival hemorrhages, ciliary injection, and hypopyon. Iris appeared yellow and covered with exudate. Right eye normal. Rabbit killed with ether. Hemolytic streptococci in cultures from purulent content of anterior chamber. Streptococci in cultures from joint. Heart blood sterile.

Fig. 1. Severe suppurative iridocyclitis in Rabbit 3. Note the exudate in the anterior chamber, and the swollen infiltrated iris and ciliary body, 3 days after intravenous injection of streptococci from the patient.   × 10.

Rabbit 4.—May 19, intravenously injected with a suspension of one-half blood-agar-slant transfer from the culture used for Rabbit 1.

May 20.—Eyes unchanged.

May 21.—Alive but condition not recorded.

May 22.—Found dead. Left eye showed conjunctival hemorrhage; limbal vessels were injected; palpebral conjunctiva red, hemorrhagic. Iris not satisfactorily examined because of clouded cornea. The clinical observations of this animal were hardly sufficient for a diagnosis of iridocyclitis. Sections, however, showed lesion of the ciliary body, with many streptococci in the tissue of the corona ciliaris. Right eye normal. Streptococci in culture from knee joints. A small subendocardial hemorrhage; valves normal. No other gross visceral lesions. No herpes.

A suspension of fluid containing streptococci from the eye of Rabbit 3 was injected intravenously into Rabbit 7. No changes were observed in the eyes and the animal was discharged apparently well 26 days later. The number of streptococci injected was necessarily small. Pure cultures of streptococci from the eye of Rabbit 3 (suspended in salt solution, the amount being equivalent to 3 c.c. of an 18-hour culture in ascites dextrose broth) were injected intravenously into Rabbits 8 and 9. No ocular lesions were produced.

Series II

The cultures used in this series were isolated from the patient May 24. At this time the iritis was worse than it had been for some days previously, and there was severe inflammation in the tear sac.

Four rabbits (10, 11, 12, 13) were injected with suspensions of 20-hour ascites-dextrose-broth cultures inoculated directly from the patient. These cultures contained a small number of staphylococci in addition to the pre-dominating streptococci. Rabbit 11 died 4 days later without evidence of iritis. There were no clearly marked focal lesions in the viscera; cultures from the heart blood and from the joints showed streptococci. Rabbit 13 showed no iritis for 4 days; was found dead on the fifth day. The media of the left eye were more cloudy than usual, but stained sections showed no evidence of iritis. There was a large pulmonary infarct. Rabbits 10 and 12 developed definite iritis.

Rabbit 10.—May 25, received a suspension of organisms from 5 c.c. of an ascites-dextrose-broth culture. After 24 hours, conjunctivitis developed in the right eye. Iris dull diffuse red in color. Four hours later conjunctivitis more marked, with edema of lids and lacrimation; ciliary injection; "iris covered with spots of exudate"; the color of the iris at this time, more gray-yellow. At 28 hours, the aqueous was clouded. Left eye normal. Animal lively. At 48 hours there were severe conjunctivitis, ciliary injection, chemosis. Iris beaded with exudate, the pupil partly obliterated by a circular gray-white exudate adherent to the iris margin. Spontaneous perforation occurred at the corneal margin, and death 52 hours after inoculation. The exudate from the anterior chamber contained gram-positive cocci in chains; hemolytic strepto-cocci were shown in cultures from the anterior chamber, from the heart blood and from the knee joint. No herpes; no gross changes in muscles, appendix, intestine, liver, spleen, or kidney. In the lungs some edema. Several small hemorrhages in the stomach. One small subendocardial hemorrhage in the right auricle; the valves normal.

Rabbit 26.—May 28, received a suspension of streptococci (from 6 c.c. of a 24-hour culture in ascites dextrose broth) isolated from the eye of Rabbit 10. After 24 hours, the left eye showed slight lacrimation, with injec-tion of conjunctival vessels, marked ciliary injection, and cloudy aqueous. The cornea perforated spontaneously 72 hours after inoculation, and the animal was found dead on the 4th day. Streptococci shown in cultures from aqueous, heart blood, and knee joint. No significant visceral lesions were found.

Rabbit 26a.—Inoculated with a smaller amount of the same culture that Rabbit 26 had received, died on the 4th day. No lesions of the eyes or of the viscera found. Streptococci shown in the heart blood.

Streptococci from 2 c.c. of an 18-hour ascites-dextrose-broth culture from the eye of Rabbit 26 were injected intravenously into 4 rabbits (40, 41, 42, 43). One animal died after 4 days. Cultures from the heart blood showed no strepto-cocci. The other 3 rabbits were discharged well after 14 days.

Streptococci from the heart blood of Rabbit 26 injected into 2 rabbits (37, 38) produced no definite lesions of the eyes. In one animal (37), dead on the 6th day, the vessels of the left nictitating membrane were more prominent (probably a result of the position of the animal after death). The joint fluid was cloudy; the colon bacillus was isolated. No streptococci were found in the heart blood. Rabbit 38 developed severe arthritis but survived. It was killed with chloroform 8 weeks after inoculation. Cultures from heart blood and joints remained sterile. There were no visceral lesions except moderate coccidiosis of the liver.

Rabbit 12.—Received a suspension of streptococci from 5 c.c. of a 20-hour ascites-dextrose-broth culture on May 25. After 48 hours the eyes appeared normal, but after 72 hours the left eye was inflamed with marked conjunctivitis, chemosis, and lacrimation. The iris (normally brown) appeared hazy, gray-yellow in color, with an adherent exudate. Hypopyon, with perforation, was followed by ocular hemorrhage and the death of the animal on the 5th day.

There were found a few small hemorrhages in the mucosa of the stomach, and a subendocardial hemorrhage 2 mm. in diameter in the right auricle. No other gross visceral lesions.

Cultures from within the left eye showed hemolytic streptococci. Streptococci were shown in cultures from the heart blood. Sections showed inflammation of the ciliary body, with exudate and masses of streptococci in the anterior chamber.

Four rabbits (33, 34, 35, 36) received suspensions of a pure culture of a hemolytic streptococcus from the eye of Rabbit 12.

Rabbit 36.—The left conjunctiva was injected and there was some lacrimation 24 hours after injection. The animal was found dead on the second day. The anterior chamber was cloudy (possibly postmortem change). Cultures from the heart blood and from the left knee joint showed streptococci.

Rabbits 33, 34 and 35.—Died after 1, 7, and 16 days; streptococci were shown in cultures from the heart blood of 33 and 34, and from the knee joint of 33. There were no ocular lesions.

Rabbits 16 and 17.—Injected with a 3rd transfer in culture from the patient; showed no lesions of the eyes.

Series III

Cultures taken May 26 from the tear sac of the patient, after curettage and packing 4 hours previously, contained hemolytic streptococci and a few staphylococci. To avoid as far as possible changes in the organisms during the period of subculture, the original broth cultures containing staphylococci, together with streptococci, were centrifugated and the animals injected with sedimented organisms within 24 hours after removal from the patient.

Two rabbits (18, 19) died after 12 and 11 days. There were no ocular lesions.

Series IV

Cultures taken May 28 from the tear sac were similar to those of Series III. Two rabbits (30,31) died after 3 and 15 days. There were no ocular lesions. Streptococci were found in the joint of Rabbit 30.

Series V

Cultures taken June 2 from the tear sac contained chiefly hemolytic streptococci, with a few staphylococci.

Four rabbits (44, 45, 46, 47) were injected with pure cultures of the strepto-
cocci. Two survived without lesions of the eyes (49, 50). In Rabbit 51, there
was no visible lesion of the eye on the 2nd day; on the 3rd day the animal
was found dead. The conjunctiva of the left eye was moderately injected,
with some exudate, but there was no clearly defined lesion of the
bulb (probably a postmortem change or due to terminal struggles). Sections
showed no lesions of the uveal tract. Cultures from the turbid joint fluid con-
tained streptococci. Rabbit 48 died on the 15th day without ocular lesions.

SERIES VI

Cultures from the tear sac, June 7, showed an almost pure culture of a
hemolytic streptococcus.

Six rabbits (55-60) injected with these cultures developed no lesions of
the eye.

SERIES VII

Cultures from the tear sac, June 16, yielded streptococci and a few
staphylococci.

Four rabbits (81-84) were injected with doses corresponding as nearly
as possible to those given to the rabbits in Series II. Three of the 4 succumbed
within 3 days, and hemolytic streptococci were isolated from the heart blood
of each. The virulence of the streptococcus for rabbits seemed not appreciably
decreased, but no definite lesions of the eyes were produced. A transient
conjunctivitis with discharge was noted in the left eye of Rabbit 81, but such
slight lesions were occasionally noted in uninoculated animals.

ANATOMIC STUDY OF THE RABBITS' EYES

As noted, 4 rabbits were injected intravenously with the pure cul-
tures of the hemolytic streptococcus obtained from the acutely inflamed
tear sac of the patient on April 28, 1915; 3 of these rabbits developed
iritis. Similarly, 2 of 4 rabbits gave positive results with material
obtained 26 days later (Series II). Four of these animals showed
clinical and anatomic suppurative iridocyclitis; in Rabbit 4 the lesion
did not reach the suppurative stage before the death of the animal.
The streptococcus was found inside all the affected eyes and not in
the fellow eyes.

Rabbit 3 of the first series was typical.

On section the episclera was found much infiltrated and the sclera at the
sulcus permeated by cells through all layers. The corneal epithelium was
absent for the most part and where present at all, was reduced to 1 or 2 layers.
The anterior two-thirds of the stroma was moderately infiltrated with leuko-
cytes; the posterior third was necrotic. Descemet's membrane was everywhere
denuded of its endothelium. The iris and the ciliary body were much swollen
and densely infiltrated with cells and fibrin. This exudate was breaking
through the ciliary processes and the posterior layers of the iris, filling out
the posterior chamber, throwing the ciliary processes forward, matting them
together, and binding both the corona and the iris to the lens capsule, block-
ing the pupil completely and extending over the iris through the lateral thirds
of the anterior chamber. The central pupillary third of the anterior chamber
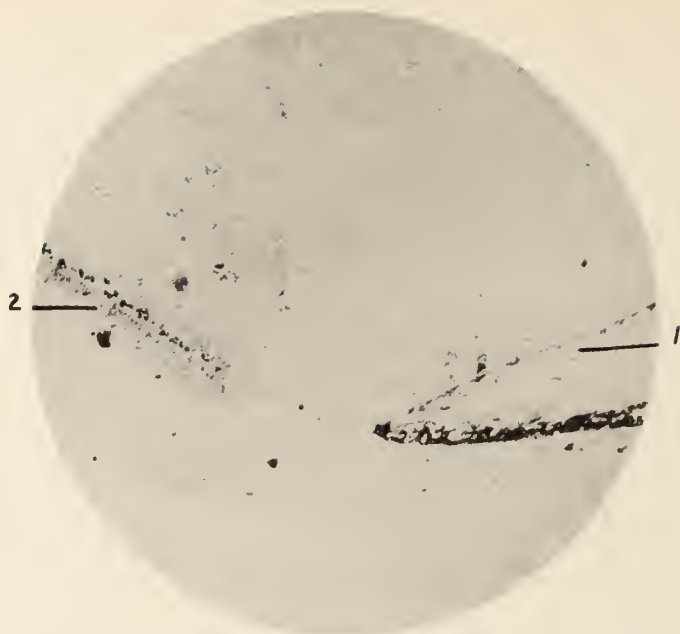
Fig. 2. Early stage of iridocyclitis in Rabbit 1, killed 5 days after inoculation. 1 = anterior surface of the iris. 2 = line of leukocytes in the pupillary area full of streptococci. × 60.
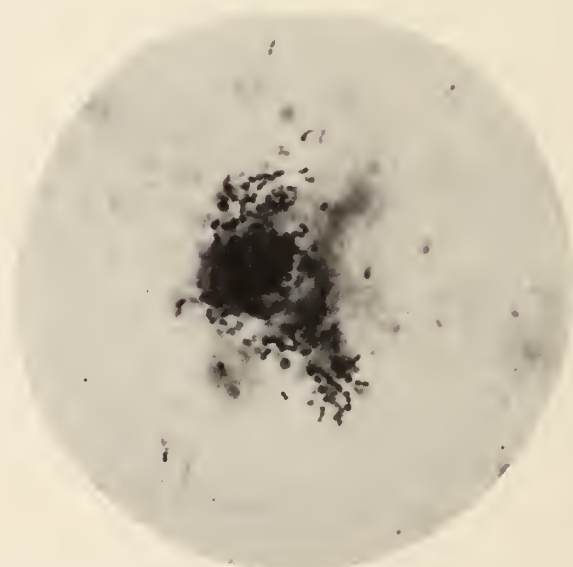
Fig. 3. Leukocyte (from the line of cells lying across the pupillary space in Fig. 2) filled with streptococci. × 1200.

was occupied by a fibrin clot. The spaces of Fontana were distended and the posterior end of Descemet's membrane was separated from the stroma by leukocytes. The exudate continued back behind the root of the iris between the ciliary body and the sclera to the beginning of the chorioidea. The chorioidea itself was little invaded and the retina not at all.

The process was therefore a severe suppurative iridocylitis. A similar but less severe iridocyclitis was found in Rabbit 1. It evidently represented an earlier stage of the process; the disposition of the organisms in the tissues could be better studied than in the severer type.

There was a sharp line of demarkation between the fibrinous clot occupying the central third of the anterior chamber and the cellular exudate which lay over the iris on each side of the pupil. Between the two was an almost unbroken row of leukocytic clumps, so filled with organisms that in Gram-Weigert sections they made a blue line running from the pupillary border to a point half way between the center and the base of the cornea on each side. In front of the line the fibrin clot was almost, if not entirely, devoid of cocci, but behind it the exudate was everywhere richly permeated with chains and groups of streptococci. The iris itself was only moderately infiltrated, much less so than the ciliary processes, and no organisms were found in the iris. Other sections showed 2 fairly large abscesses in the posterior portion of the ciliary body, just external to the line of the bases of the processes. The abscesses contained no visible organisms. The exudate between the ciliary processes contained large numbers of cocci, yet they were much less numerous than in front of the iris. In contrast to conditions found in the iris numerous organisms could be found within the stroma of the ciliary processes, about the vessels, among the infiltrating cells, and between the cells making up the layers of its unpigmented and pigmented epithelium. No organisms could be found in the infiltrate blocking the spaces of Fontana and the ligamentum pectinatum, or lying outside the ciliary body. The origin of the great mass of both the leukocytes and the organisms, therefore, seemed to be in the ciliary body rather than in the iris.

Rabbit 4 showed the earlier changes produced by the invading organism.

Some sections showed a fibrin clot and a large leukocytic node in one sinus angle. This mass of leukocytes could be followed well back through the ciliary body in successive sections to the posterior end of the corona ciliaris, where it formed a node about a vein (Fig. 5). Gram-Weigert stains showed many chains and clumps of streptococci free in tissue spaces throughout the ciliary body. (Figs. 6 and 7.)

Rabbit 12 of the second series showed a more extensive suppurative process, with rupture of the sclera clinically.

On section a broad gaping rupture was found near the posterior end of the ciliary body on one side, with hemorrhage and prolapse of the detached retina part way into the wound. The iris and ciliary body showed very extensive infiltration, and the pupil and both chambers were choc-a-bloc with fibrin
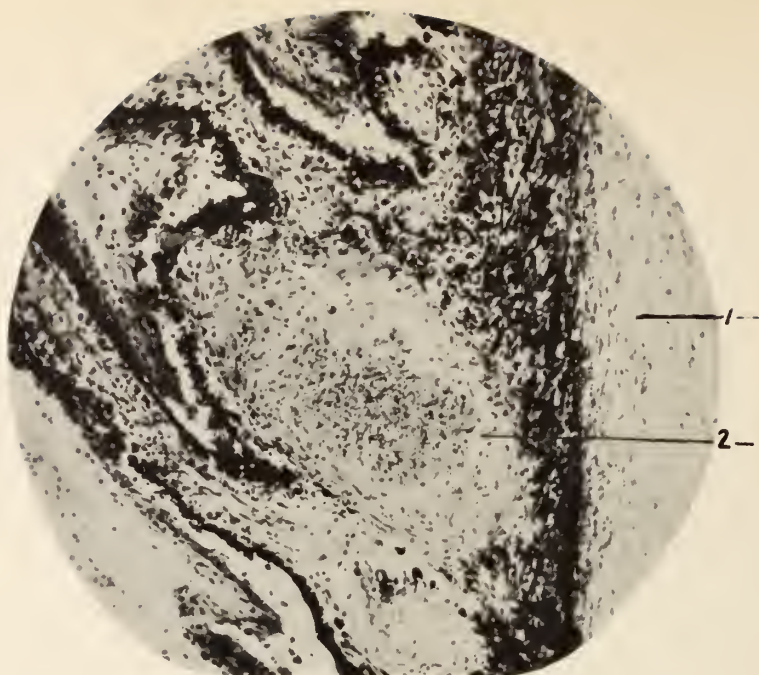
Fig. 4. Abscess in the posterior part of the ciliary body in Rabbit 1. 1 = sclera.
2 = ciliary body with abscess. × 140.
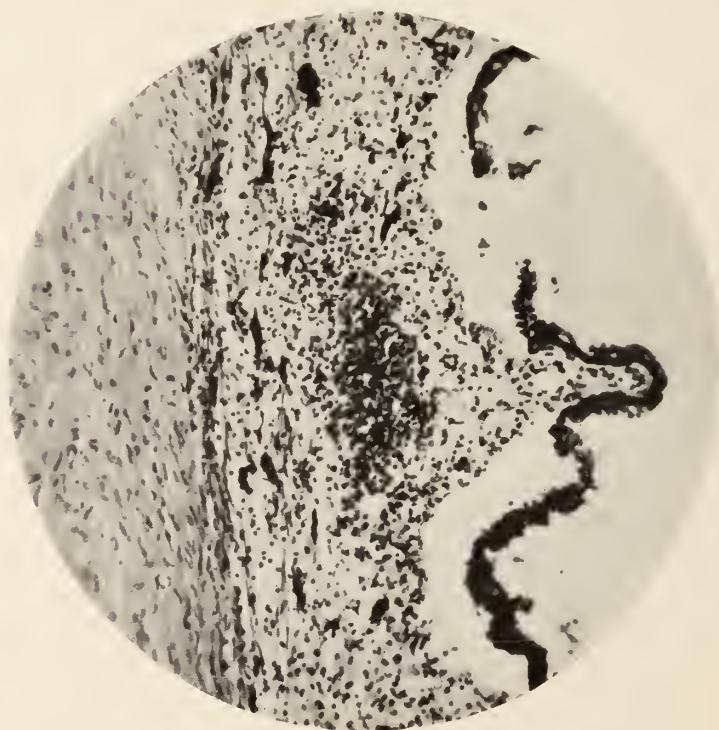
Fig. 5. Node from the infiltration about a vein in the posterior part of the ciliary body.
× 170.

and a dense leukocytic exudate. The iris and the ciliary body were similar in appearance to those of Rabbit 3. The marginal thirds of the cornea were swollen to twice the normal thickness. There were an almost complete loss of corneal epithelium and considerable infiltration of the anterior third of the stroma. The region of the ligamentum pectinatum and of the canal of Schlemm showed a massive infiltration, extending half way back to the ora and detaching the ciliary body from the sclera. The iris was bound to the lens on one side by flat synechiae and by synechia of the pupillary border on the other side. The body of the iris on this side was thrown forward, its root adherent to the base of the cornea. The retina and chorioidea were both normal.

Rabbit 10 showed essentially the same anatomic findings. Rabbit 26 (inoculated with pure cultures obtained from the aqueous of Rabbit 10) showed a clinical suppurative iridocyclitis. Most of the eye was sacrificed for cultural purposes, but the organisms were found in sections of the uveal exudate.

Sections from the eyes of 20 other injected rabbits in which there were no clinical evidences of ocular inflammation were carefully examined for inflammatory lesions and bacteria, with negative results.

SUMMARY OF SERIES I TO VII

Hemolytic streptococci isolated from the tear sac of the patient, April 28, during a period of acute iridocyclitis produced iridocyclitis with later hypopyon and perforation of the bulb in 2 of 4 rabbits, and a less marked lesion in Rabbit 4 (Series I). These cultures were from the 3rd and 4th transfers on blood-agar slants. Cultures taken from the tear sac on May 24 during a period of exacerbation of the inflammation in the eye and the tear sac produced iridocyclitis in 2 of 4 rabbits. Cultures of hemolytic streptococci taken from the tear sac on 5 subsequent occasions (May 26 to June 16), during a period of continued improvement in the condition of the eye and the tear sac of the patient, produced no lesions of the eye in a total of 22 rabbits. These cultures were inoculated either from the 1st transfer from the patient (in this case staphylococci were sometimes present), or from the 2nd transfer in pure culture after isolation on blood-agar plates.

The failure of the later cultures from the patient to produce iridocyclitis in rabbits, did not seem to be associated with an appreciable decrease in virulence for rabbits. When equivalent quantities of streptococci were inoculated, animals in the later series died in about the same time as those of the earlier series, and hemolytic streptococci were isolated usually from the heart blood, and frequently from the

Fig. 6. Masses of the streptococci in the ciliary body in Rabbit 4. 1 = pigment epithelium of the ciliary body. 2 = stained masses of organisms in the loose structure of the ciliary body. Gram-Weigert. × 170.
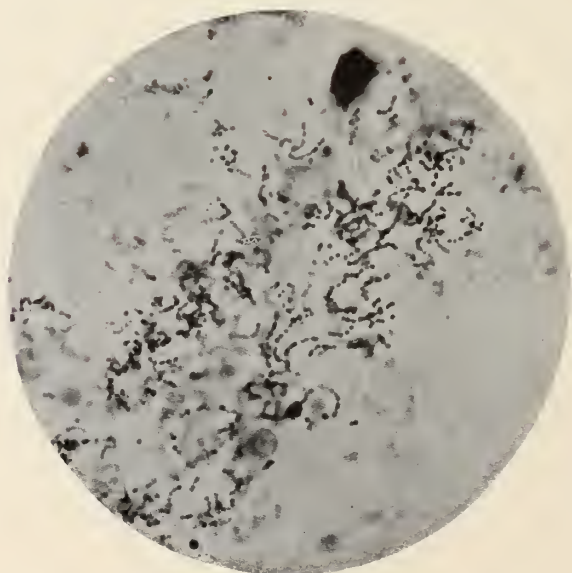
Fig. 7. Streptococci from the area marked "2" in Fig. 6 (Rabbit 4). × 1200.

joints. Other gross visceral lesions were not frequent, tho small hemorrhages in the endocardium, pleura, stomach, and infarcts of the lung, were occasionally noted.

Cultures which had produced ocular lesions in rabbits, and had then been kept in the incubator for 10 days (2 subcultures) no longer produced these lesions. The strain which had produced iridocyclitis in Rabbits 1 and 3 was kept at 37 C. for a few days, and then placed in the ice box. July 7 (52 days after isolation) subcultures no longer produced ocular lesions in rabbits.

Attempts were made to produce lesions of the eye from rabbit to rabbit either by direct transfer of the exudate from the eye or by means of one intervening subculture. Thus, cultures of the streptococcus from the eye of Rabbit 10, in which iridocyclitis had been produced by intravenous inoculations, were injected intravenously into Rabbit 26. Iridocyclitis was produced in this animal and hemolytic streptococci were isolated from the eye and from the heart blood, but inoculations of these cultures in turn in 6 more rabbits failed to produce any further iritis.

The power of the hemolytic streptococcus in these experiments, to localize in the eye and produce iridocyclitis in rabbits was lost in a relatively short time (1) in the body of the original host (tear sac), (2) in cultures, and (3) during animal passage. This change in tissue localization seemed not to be associated with any demonstrable decrease in lethal power.

The hemolytic properties and fermentative reactions in various sugar media of the streptococci from the tear sac were studied from time to time after growth on culture media and after successive passages through animals. The zone of hemolysis on blood-agar plates became somewhat smaller after 2 months growth on culture media (blood agar). After 6 months the 11th subculture showed no further decrease in the zone of hemolysis. When first isolated, cultures formed acid in media containing dextrose, saccharose, lactose, or salicin, but not in media containing raffinose, mannite, or inulin. In later cultures (2 months), mannite and raffinose were inconstantly fermented. After passage through a rabbit (intraocular injection), the streptococcus, being recovered in pure culture, when placed in sugar media again gave the same reactions as when first isolated from the patient.

The failure of the later cultures to produce lesions in the eyes of rabbits did not appear to be accompanied by any constant alteration in fermentative power. Some decrease in hemolytic power was noted.

THE EFFECT ON STREPTOCOCCI OF RESIDENCE IN THE EYE

The idea that organisms which ordinarily do not localize in a given tissue may be so modified by continued residence and growth in it that they later localize in the unusual site with more or less regularity, is by no means a new one.

Bezançon and Labbé[2] inoculated an animal intravenously with staphylococcus from a cutaneous abscess, and after traumatizing a joint, obtained localization in the joint. Cultures obtained from this joint showed a tendency to localize in joints on subsequent intravenous inoculation into animals, altho this localization could not be maintained in subsequent series of animals. Forssner[3] (1902), working with a streptococcus isolated from an axillary abscess, grew the organism in extracts of kidney and in kidney tissue both in vitro and in vivo. Whereas the original organism showed no tendency to localize in the kidneys on intravenous injection, the strains obtained after passage through kidneys of animals exhibited a remarkable tendency to produce lesions of the kidneys on intravenous injection. This property after being acquired, was retained by the organism for 4 or 5 generations on serum broth after the last animal passage, and then was lost.

Other organisms behave similarly. Strains of B. pestis repeatedly grown in the lung show an increased tendency to produce pneumonic lesions on intravenous, or even subcutaneous, injections.

The experiments of Forssner in which organisms after being grown in a special organ of the body, such as the kidney, acquired the power to localize and produce lesions with increasing frequency in that organ after intravenous injection, suggested the possibility of producing by successive transfers from eye to eye in rabbits a similar localization. It is obvious that the streptococcus from our patient did not acquire its quality of localization through actual previous residence in the tissues of his eye, and even if we admit the possible influence of residence in a contiguous organ (tear duct), this relation could hardly be held to explain the course of events in other cases in which ocular or other metastases originate from primary bacterial lesions in distant parts of the body. Nevertheless, it seemed desirable to find whether an organism which had previously produced iridocyclitis in rabbits could regain this quality of localization by continued growth in the tissues of the eye.

1. The strain of hemolytic streptococcus which had produced ocular lesions in Rabbits 1, 3, and 4, after being kept on blood agar for about 2 months in the ice box, no longer produced lesions of the eye. Cultures

[2] Compt. rend. Soc. de biol., 1900.
[3] Nord. Med. Ark., 1902, Part 2, No. 4, p. 18.

were inoculated into the anterior chamber of the eye of a rabbit; after 2 days, cultures from the eye were made in ascites dextrose broth, and after 18 to 24 hours' growth in incubator, a portion of this culture was introduced into the eye of a second rabbit. This transfer was repeated for 7 animal passages. After each eye passage the organisms from the broth cultures, centrifugated and suspended in salt solution, were injected intravenously into from 2 to 4 rabbits. In the later transfers cultures from the eye contained in addition to hemolytic streptococci, staphylococci, and still later non-hemolyzing streptococci in large numbers. In order to minimize the period of growth outside the eye in this experiment, the hemolytic streptococci were not isolated in pure culture before injection into the succeeding eye, but the relative proportions of streptococci and staphylococci were determined for each culture inoculated.

No ocular lesions were produced in the 14 rabbits injected during the first 5 eye passages. In cultures from the 6th and 7th animal passages most of the colonies of streptococci no longer produced hemolysis on blood-agar plates. One rabbit (196, injected with cultures from the 7th eye passage) showed some circumcorneal injection of both eyes, but sections showed no lesions of the uveal tract.

From a comparison of the non-hemolyzing streptococcus with pure cultures of the original strain of hemolytic streptococcus we are inclined to think that the decrease in number of hemolytic colonies, and increase in non-hemolytic colonies was due to the introduction during animal passage of a contaminating streptococcus which happened to be non-hemolytic, rather than that the original hemolytic strain underwent any sudden change in characteristics with respect to growth on blood agar.

The animals injected intravenously with cultures from the later eye passages (4th to 7th) showed increasing lesions of the kidney and of the heart muscles (miliary abscesses). Cultures and sections demonstrated, however, that staphylococci and not streptococci were present in these lesions; comparative counts of streptococci and staphylococci in the injected cultures showed an increase in the proportion of staphylococci where abscesses were most numerous.

2. A strain of hemolytic streptococcus taken from the tear sac after subsidence of iritis (Series VII), which had failed to produce lesions of the eye in rabbits when first isolated, was similarly passed through 7 eyes. Hemolytic streptococci disappeared from the cultures

after the 5th eye passage. The streptococci remaining in cultures were uniformly non-hemolytic and otherwise appeared culturally different from the original. Of 10 rabbits receiving hemolytic streptococci after the first 4 eye passages, and 9 rabbits from the later 3 passages, none developed characteristic lesions of the eye. One rabbit injected with cultures from the 7th eye passage showed a slight circumcorneal inflammation for 2 days, but no signs of further intraocular inflammation developed and the animal was discharged after 3 weeks.

3. A laboratory strain of hemolytic streptococcus (from severe tonsillitis) was similarly passed through a series of 7 rabbit eyes, and injected intravenously into rabbits after each of the last 4 passages. Cultures from the 7th eye passage contained both hemolytic streptococci corresponding culturally to the original strain, and non-hemolytic streptococci growing in small dry colonies, resembling those observed in the cultures from later passages of the two preceding series. Pure cultures of both types were injected into rabbits. No ocular lesions were produced in a total of 20 rabbits injected in this series.

The appearance of the non-hemolyzing type of streptococcus in cultures from the later injected eyes, raises the question as to whether this organism was a derivative of the original hemolyzing strain, or was a contamination introduced during animal passage. That opportunities for such contamination were repeatedly present, is evident from the conditions of the experiment, and the appearance of staphylococci in the later cultures. The non-hemolyzing small gray colonies of streptococcus became more numerous in cultures from the later animal passages in all 3 series of rabbits. In 2 of these series strains of streptococci from the tear sac of the patient were used, while in the 3rd series a laboratory culture of a hemolytic streptococcus from tonsillitis was employed, and yet the non-hemolyzing type appeared at about the same time in all 3 series.

The colonies of the non-hemolyzing organisms were smaller and dryer than those of the original hemolytic strains. Raffinose, mannite, and inulin were fermented, as well as dextrose, saccharose, lactose, and salicin, whereas the 3 original hemolytic strains fermented regularly dextrose, saccharose, lactose, and salicin, and only occasionally raffinose, or mannite, over a period of 6 months, both before and after animal passage.

Intraocular injections offer greater opportunities for contamination than intravenous, and so it is hardly fair to compare the appearance

of a new type of streptococci in these experiments with the changes reported in cultures of streptococci after animal passage following intravenous inoculation. However, the sudden appearance of the new type of streptococcus in all 3 series of inoculations, which seems in this instance best explained as a contamination derived from the animals, emphasizes the need of care in the interpretation of results, when strikingly new cultural characteristics appear suddenly during animal passage.

SUMMARY

The left eye of a patient suffering from a chronic dacryocystitis became acutely inflamed (iridocyclitis), April 11, coincidently with an acute exacerbation of the inflammation of the tear sac.

Hemolytic streptococci isolated from the tear sac April 28, produced typical iridocyclitis in 3 of 4 rabbits injected intravenously. Hemolytic streptococci isolated from the tear sac May 24, produced iridocyclitis in 2 of 4 rabbits injected. Cultures of hemolytic streptococci from the tear sac taken on May 26, May 28, June 2, June 7, and June 16, failed to produce iridocyclitis in a total of 22 rabbits, whether injected with other organisms from broth culture inoculated directly from the patient, or in pure culture after isolation on blood agar. That the failure of later cultures to produce iritis was not fortuitous, but more probably due to some change in the invasive power of the organism for ocular tissue seems evident from the fact that whereas in the first 2 series, 5 of 8 rabbits injected developed iridocyclitis, in the later 5 series of cultures 22 rabbits showed no such lesions. Furthermore, in the later experiments 24 other rabbits received the same streptococcus either in pure culture or with other organisms, without the production of typical iridocyclitis, such as was observed in the first 5 rabbits. The ability of the cultures to produce lesions of the eye was lost also in transfer from rabbit to rabbit, altho iritis was produced in Rabbit 26 by intravenous injection of the hemolytic streptococcus from the eye of Rabbit 10. This loss of power to produce iritis in rabbits was not accompanied by any demonstrable decrease in virulence for rabbits.

In cultures the quality of localization in the eye was lost after the 3rd or 4th subculture, usually within very few days after isolation. In the case of the first rabbits injected, however, cultures produced iritis 17 days after isolation from the patient.

Attempts to obtain a return of invasive power for tissues of the eye in strains of streptococci which had lost it, by growing the organisms in the living eye, were unsuccessful in one series after passage through 5 animals, and in one series after passage through 7 animals.

These experiments seem to indicate that the invasive power of an organism for special tissue may change within a short period of time during residence in the original host, during animal passage, and in culture, without pronounced or constant changes in cultural characteristics, or in general virulence for animals.

THE TUBERCULOCIDAL ACTION OF ARSENIC COMPOUNDS AND THEIR DISTRIBUTION IN THE TUBERCULOUS ORGANISM *

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS, XIV

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In the course of the study of problems relating to the chemotherapy of tuberculosis, we have investigated the value of arsenic compounds asserted by many clinical observers to be of definite value in the treatment of tuberculosis, and determined (1) the bactericidal action of arsenic on the tubercle bacillus, and (2) its distribution in the tuberculous organism.

HISTORICAL REVIEW

Arsenic has been used in the treatment of tuberculosis since ancient times. Dioscorides employed it internally and by inhalation. Antylus, who lived in the third century A. D., Marcellus Empyricus, and Galen all recommended it and described cures from the inhalation of powdered arsenic. It was also employed by the Chinese, and thence by the Hindus, who were acquainted not only with the oxid but also with the sulfur compound, realgar. A complete and interesting historical sketch of the use of arsenic in therapeutics, especially in tuberculosis, has been published by Kock.[1]

In a recent study of the action of arsenic in tuberculosis Burow[2] found that potassium arsenite prevented the growth of tubercle bacilli in vitro. He further held that rabbit blood serum from an animal treated with guaiacol-arsenic (sodium and potassium guaiacolate each 1.5% with 0.01% potassium arsenite) prevented the growth of tubercle bacilli. This combination was given to tuberculous rabbits and guinea-pigs with, it is asserted, specific effect, altho the treated animals showed evidence of tuberculosis. The work of Burow cannot be said to have proved a specific action of the arsenic if one considers the variations which occur after inoculation of animals of the same species. The results of Burow have been contradicted by Nurnberger,[3] who observed no specific action in tuberculosis, or on the tubercle bacilli in vitro.

Many clinical investigators have failed to demonstrate any specific effect of arsenic compounds in tuberculosis, among them being Knothe,[4] Plicque,[5] Grodecki[6] (arsenical serum), Darthenay[7] (arrhenal), Jacobi[8] (arsenous acid

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[1] Nord. Med. Ark., 1902, 35, Afd. 2.
[2] München. med. Wchnschr., 1910, 57, p. 1792.
[3] Ibid., 1911, 58, p. 2669.
[4] Wien. klin. Wchnschr., 1911, 24, p. 562.
[5] Jour. de méd. int., Paris, 1904, 8, p. 294.
[6] Przegl. lek., Krakow, 1897, 36, p. 558.
[7] Contribution à l'etude de l'action de l'arrhenal sur la nutrition des tuberculeux, 1912.
[8] New York State Jour. Med., 1912, 12, p. 358.

with digitalis), Jolly,[9] Schmey,[10] Hoff[11] (arsenic cinnimate), and Vigenaud[12] (disodium methyl arsinate). Cybulski,[13] using sodium arsenite, found no specific action in pulmonary tuberculosis. Bayeux[14] found sodium cacodylate a valuable adjuvant in infancy and adolescence. M. Levrat[15] and G. Levrat,[16] after using sodium cacodylate in over 1,000 patients, concluded that it is not a panacea for tuberculosis, but that in certain cases it is an adjuvant of incontestable value. In fever the drug may be harmful. The findings of Kock,[1] Decombins,[17] Jalaguier,[18] Nicati,[19] Anelli,[20] and Eysséric[21] are in accord with these results.

Atoxyl, used with tuberculin by Mendel,[22] and with ichthyol salicylate by Rohden,[23] was asserted by them to have a specific action on the tubercle bacillus, but they gave no experimental proof. They even concluded that salvarsan might be more specific. Renon and Delille[24] found no favorable effect in any forms of tuberculosis in human cases, or in tuberculous guinea-pigs.

That salvarsan has no specific action can be seen from the report of M. Bernay and A. Bernay,[25] whose results do not show any effects of this compound on tuberculosis. Lundie and Blaikie,[26] using sodium para-aminophenylarsonate (seomin), which contains 22.8% arsenic, found that it was not a cure for tuberculosis when given in deep injections.

To summarize the clinical reports in the literature, we may conclude that arsenic has not been demonstrated to have any specific action in tuberculosis, and that its value in this disease can be attributed only to its favorable effects on metabolism. Furthermore, in advanced tuberculosis it may even be harmful.

In order further to determine the effect of arsenic compounds on the tubercle bacillus, and their value in the treatment of tuberculosis, we have investigated the tuberculocidal action of sodium arsenite, sodium cacodylate, mercury cacodylate, atoxyl, arsacetin, and neosalvarsan, and have sought to determine whether or not these compounds enter the tissues of the tuberculous animal. A chemical substance to

[9] Jour. de méd. de Paris, 1902, 14, p. 277.

[10] Aertzl. Centr.-Anz., 1899, 11, p. 547.

[11] Ibid., 1899, 11, p. 530.

[12] Bull. gén. de thérap., 1902, 143, p. 920.

[13] München. med. Wchnschr., 1902, 49, p. 1393.

[14] Cong. Internat. de la Tuberc., 1905, Paris, 1906, 2, p. 368.

[15] Lyon méd., 1905, 104, p. 449.

[16] Rev. de méd., 1911, 31, p. 493.

[17] Le cacodylate de soude à doses massives et espacées dans le traitement de la tuberculose pulmonaire, 1914.

[18] Le cacodylate de soude dans la tuberculose pulmonaire et quelques autres affections, 1901.

[19] Le cacodylate de soude dans la tuberculose pulmonaire (Etude therapeutique et pharmacodynamique), 1902.

[20] Riforma med., 1901, 17, p. 196.

[21] Traitément de la tuberculose pulmonaire par le cacodylate de strychnine à hautes doses, 1902.

[22] München. med. Wchnschr., 1909, 56, p. 13.

[23] Memoranda medica, 1903.

[24] Bull. gén. de thérap., 1907, 154, p. 7.

[25] Jour. de méd. de Paris, 1912, 24, pp. 653, 975.

[26] Brit. Med. Jour., 1910, 1, p. 196.

be of value in tuberculosis must not only possess a specific germicidal or inhibitory action on the tubercle bacillus, but it must be able to permeate the avascular tubercle or be deposited there in sufficient concentration to exert its specific action. These problems have been discussed in the previous articles by Wells, Corper, and others.

ACTION OF ARSENIC COMPOUNDS ON TUBERCLE BACILLI IN VITRO

For determining the tuberculocidal action of arsenic compounds an emulsion of human tubercle bacilli was treated at 37 C. with various dilutions of each arsenic substance for a period of 24 hours. This length of time was adopted that we might ascertain from the beginning the value of these compounds as tuberculocidal agents; for a compound which is unable to cause death of the tubercle bacilli in 24 hours under favorable conditions could hardly be expected to be of value in such unfavorable conditions as obtain in the organism. If the arsenic compounds are unable to kill the tubercle bacilli in 24 hours, then injection of these treated bacilli will produce tuberculosis in the guinea-pig. If tuberculosis is not produced, then the compound has either killed the tubercle bacilli or inhibited their growth. In the latter case it is necessary to remove the chemical agent or neutralize its action before injection into animals.

In all the tests, results of which are given in Tables 1 to 6, the same technic was employed. To each tube containing 5 c.c. of solution were added 3 drops of a heavy suspension of human tubercle bacilli. These tubes were incubated at 37 C. for 24 hours. At the end of this time the bacilli were centrifugated and washed with salt solution and then injected subcutaneously into the inguinal region of normal guinea-pigs.

The Tuberculocidal Action of Sodium Arsenite.—We see from Table 1 that sodium arsenite in dilutions of from 0.1% to 0.0001% has no germicidal action on the tubercle bacillus in vitro in 24 hours at 37 C. All the animals showed evidence of generalized tuberculosis, and the differences were such as are expected in any series of inoculated animals. There is not even evidence of any inhibitory effect on the human tubercle bacillus.

The Tuberculocidal Action of Sodium Cacodylate.—Table 2 shows that sodium cacodylate in dilutions of from 2% to 0.002% has no germicidal action on human tubercle bacilli in vitro. In this series all the animals developed generalized tuberculosis, altho the animals injected with tubercle bacilli treated with the more concentrated solutions lived longer.

TABLE 1

THE TUBERCULOCIDAL ACTION OF SODIUM ARSENITE

Dilution of Sodium Arsenite	Duration of Life	Liver	Spleen	Lungs	Local Lymph Glands	Miscellaneous
0.1%	172 days..	Multiple foci of miliary tubercles	Enlarged; few miliary tubercles	Miliary tubercles; hemorrhagic	Large gland 2 cm. in diameter; local ulcer	Retroperitoneal glands caseous
0.01%	84 days..	Miliary tuberculosis	Numerous tubercles	Appear normal	Large tubercle 2 cm. in diameter	
0.001%	116 days..	Necrotic caseous areas	Five times normal size; necrotic caseous areas	Few miliary tubercles	Large local gland; local ulcer	Retroperitoneal and peribronchial glands enlarged
0.0001%	62 days..	Multiple tubercles and areas of necrosis	Greatly enlarged; necrotic caseous areas	Tubercles throughout; hemorrhagic	No enlarged local or retroperitoneal glands	Tubercular peritonitis

The kidneys were normal.

TABLE 2

THE TUBERCULOCIDAL ACTION OF SODIUM CACODYLATE

Dilution of Sodium Cacodylate	Duration of Life	Liver	Spleen	Lungs	Local Lymph Glands	Miscellaneous
2%	Killed after 152 days	Necrotic tuberculous areas	Enlarged miliary tubercles	Numerous tubercles	Caseous, greatly enlarged	Retroperitoneal and peribronchial glands enlarged
0.2%	152 days..	Numerous necrotic areas	About 6 times normal size; largely necrotic	Few small tubercles	Large ulcer; caseous glands	Peribronchial glands caseous; retroperitoneal glands enlarged
0.02%	137 days..	Miliary tubercles	About 10 times normal size; entirely necrotic	Small tubercles; hemorrhagic	Enlarged, caseous	Peribronchial and retroperitoneal glands enlarged
0.002%	56 days..	Several large necrotic areas	About 10 times normal size; entirely tuberculous	Numerous tubercles from 1 to 5 mm. in diameter	Large tubercle; caseous	Enlarged peribronchial glands

The kidneys were normal.

The Tuberculocidal Action of Mercury Cacodylate.—Table 3 indicates that mercury cacodylate in dilutions up to 0.001%, has a germicidal action on human tubercle bacilli.   The death of the first animal, which received the injection of tubercle bacilli treated with 1% mercury cacodylate, was undoubtedly due to the toxic action of the mercury cacodylate, which had not been entirely removed in centrifugation.   As the suspensions in this series had been centrifugated, and the upper 4 c.c. of cacodylate removed and replaced with salt solution, evidently 1 c.c. of the mercury cacodylate (1%) was toxic for the guinea-pig. The hemorrhages in the lungs, intestines, and at the site of injection were caused by the mercury cacodylate.

TABLE   3

The Tuberculocidal Action of Mercury Cacodylate

Dilution of Mercury Cacodylate	Duration of Life	Liver	Lungs	Kidneys	Local Lymph Glands	Miscellaneous
1%	3 days....	Hyperemia; centers of lobules distinct	Hemorrhagic, edematous	Pale, slightly swollen	Normal......	Subcutaneous hemorrhages at site of injection; hemorrhages in large intestine
0.1%	Killed after 140 days	Normal.......	Normal.......	Normal.......	Slightly enlarged; no caseation	
0.01%	Killed after 140 days	Normal.......	Normal.......	Normal.......	Normal	
0.001%	Killed after 140 days	Normal.......	Normal.......	Normal.......	Normal	

The spleen was normal.

The relation of the toxic dose of this drug to the amount necessary to kill the tubercle bacillus remains to be determined.   Like that of other mercury compounds, the action of mercury cacodylate in the animal's body would probably be less marked.

The fact that sodium cacodylate has no tuberculocidal action indicates that the mercury cacodylate owes its effect to the presence of the mercury in the molecule.   DeWitt and Sherman[27] have found that mercuric chlorid in dilution of 0.001% has a germicidal action on the tubercle bacillus in 24 hours.

[27] Jour. Infect. Dis., 1914, 15, p. 245.

TABLE 4

THE TUBERCULOCIDAL ACTION OF ATOXYL

Dilution of Atoxyl	Duration of Life	Liver	Spleen	Lungs	Kidneys	Local Lymph Glands	Miscellaneous
1%	Killed after 168 days	Fatty changes; few tubercles	Adherent to surrounding tissues; enlarged, caseous	Numerous miliary tubercles	Few miliary tubercles	Enlarged, caseous	Peribronchial glands greatly enlarged
0.1%	104 days	Few miliary tubercles	Enlarged; necrotic tubercles	Miliary tubercles	Appear normal	Enlarged, caseous	
0.01%	Killed after 152 days	Caseous tuberculosis	Enlarged; miliary tubercles	Few miliary tubercles	Appear normal	Enlarged; no caseation	
0.001%	Killed after 168 days	Cirrhotic; fatty changes; minute tubercles	Enlarged; numerous tubercles	Multiple tubercles	Appear normal	Enlarged, caseous	Retroperitoneal glands enlarged, caseous; mammary glands caseous

TABLE 5

THE TUBERCULOCIDAL ACTION OF ARSACETIN

Dilution of Arsacetin	Duration of Life	Liver	Spleen	Lungs	Kidneys	Local Lymph Glands	Miscellaneous
1%	Killed after 137 days	Miliary tubercles	Enlarged; caseous	Appear normal	Appear normal	Enlarged. caseous	
0.1%	143 days	Numerous tuberculous necroses	About 10 times normal size; many tubercles	Many large pearly areas of tuberculosis in all lobes	Pale; no tubercles	Enlarged, slate-colored	Retroperitoneal and peribronchial glands enlarged
0.01%	115 days	Many necrotic areas	About 8 times normal size; mostly necrotic	Numerous large necrotic areas in lower lobes; consolidated	Appear normal	Enlarged, caseous	Peribronchial and retroperitoneal glands enlarged
0.001%	Killed after 137 days	Few minute whitish spots on surface	About 2 times normal size; few tubercles from 3 to 4 mm. in diameter	Numerous miliary tubercles	Appear normal	Enlarged, caseous	Pleural cavity contains bloody fluid

The Tuberculocidal Action of Atoxyl.—Atoxyl in dilutions of from 1% to 0.001% has no germicidal action on human tubercle bacilli in vitro. All the inoculated animals developed generalized tuberculosis. The results, given in Table 4, indicate that no conclusions can be drawn regarding the specific action of a drug on one organism from its

effects on another. The fact that atoxyl is specific for certain trypano-
somes does not indicate that it will have any action on other species
of micro-organisms.

The Tuberculocidal Action of Arsacetin.—Table 5 indicates that
arsacetin in dilutions of from 1% to 0.001% has no germicidal action
on human tubercle bacilli in vitro in 24 hours at 37 C. All the animals
presented generalized tuberculous lesions.

The Tuberculocidal Action of Neosalvarsan.—Neosalvarsan, in
dilutions of from 1% to 0.001%, has no germicidal action toward human
tubercle bacilli in vitro in 24 hours at 37 C. All the animals presented
generalized tuberculosis. The statements which occur in clinical litera-
ture suggesting a specific action of this drug in tuberculosis are there-
fore without foundation. One would hardly expect neosalvarsan to
have a specific action toward the tubercle bacillus, an organism of a
different group from that of Spirochaeta pallida, with different chemi-
cal composition and different biologic properties.

TABLE 6
THE TUBERCULOCIDAL ACTION OF NEOSALVARSAN

Dilu- tion of Neosal- varsan	Duration of Life	Liver	Spleen	Lungs	Kidneys	Local Lymph Glands	Miscellaneous
1%	87 days	Numerous necrotic areas	Enlarged; mil- iary tubercles	Numerous tubercles	Appear normal	Enlarged; local ulcer	
0.1%	41 days	Several large necrotic areas	Eight times normal size; necrotic tu- bercles	Numerous tubercles from 1 to 4 mm. in diameter	Appear normal	Large, case- ous	Peribronchial and retroperi- toneal glands enlarged
0.001%	112 days	Many tu- bercles	Enlarged; ne- crotic	Appear normal	Appear normal	Enlarged, caseous	
0.001%	112 days	Many tu- berculous necrotic areas	About 10 times normal size; entirely ne- crotic	Numerous miliary tubercles; pneumon- ic	Pale, fatty; no visi- ble tu- bercles	Enlarged, caseous, pigmented	Peribronchial and retroperi- toneal glands enlarged

ANALYTIC METHOD OF ASCERTAINING THE PRESENCE OF ARSENIC
IN TISSUES OF INJECTED ANIMALS

Since in the following analyses we are dealing with small amounts
of arsenic and are interested in determining the relative rather than
the absolute amount present in the tissues, the following method,
which is accurate for small amounts and does not require a great
deal of time for performance, was used. It is a modification of the

Marsh-Berzelius method. The Sanger-Black modification of the Gutzeit method[28] was tried in control analyses on tissues containing known amounts of arsenic, but proved inadequate, being less accurate and less delicate than the following modified Marsh method, and being accompanied by numerous pitfalls when organic matter was present.

METHOD

The method to be described revealed an absolute agreement between mirrors directly marshed, with the use of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, and 30 micromilligrams of arsenic and the same amounts of arsenic carried through the entire analytic process, in each case 10 grams of liver or 5 to 10 grams of arrowroot starch being employed. All reagents used in these analyses must give no appreciable arsenic mirror; that is, all of them together carried through the steps of the analysis must not give a mirror greater than 1 mmg. of arsenic. Those used in the following analyses gave only a faint trace—far less than 1 mmg.

Ten to fifteen grams of the tissue to be analyzed are weighed into a 4- to 5-inch porcelain evaporating dish or casserole, with a watch glass cover, and 10 c.c. of 1.84 sulfuric acid added; this suffices to preserve the tissue until the organic matter can be destroyed. When the worker is ready to destroy the organic matter, the evaporating dish is put on an air bath (having a thermometer at one side) so that only a small edge of the dish projects above the bath. The bath must be in a well-ventilated hood or else proper suction apparatus must be arranged to remove the nitric acid fumes. Then, 20 to 30 c.c. of 1.42 nitric acid are added cautiously and gentle heating begun, for a rapid violent evolution of NO_2 and frothing may otherwise occur which might occasion loss of some of the material. After the first violent frothing and evolution of NO_2 have subsided, the heating may be resumed and the temperature of the air bath brought to about 160 to 180 C. With the recurrence of charring, repeated additions of from 3 to 5 c.c. nitric acid are immediately made (charring should not continue too long without the addition of nitric acid, as there must be no formation of SO_2) until the liquid no longer turns brown but remains pale yellow—due to ferric chlorid—or colorless; a few more additions of from 2 to 3 c.c. of nitric acid are then made to insure the destruction of all organic matter, and heating increased until the appearance of white sulfuric acid fumes. Since in different air baths the temperature at which this occurs varies, only an approximate temperature can be given— about 180 to 200 C.—but the best index is the first appearance of heavy white fumes. If with the occurrence of these fumes there is no more browning of the solution, it is cooled and from 10 to 15 c.c. of distilled water added, and heat applied to boil off the water until the reappearance of white fumes. Sometimes browning again occurs at this stage and then it is necessary to make a few more additions of nitric acid. Three additions of from 10 to 15 c.c. of distilled water and heating until white fumes appear, generally suffices to free the solution from nitric acid, but to make sure of this a few drops of the liquid are added to a diphenylamin sulfate-sulfuric acid test solution[29] (1 part of nitrogen in 20,000,000 parts of water gives a violet-blue color with a drop of diphenylamin sulfate in sulfuric acid followed by 2 c.c. concentrated

[28] Jour. Sociol. Chem. Industry, 1907, 26, p. 1115.
[29] Watts: Dictionary of Chemistry, Vol. 3, p. 558.
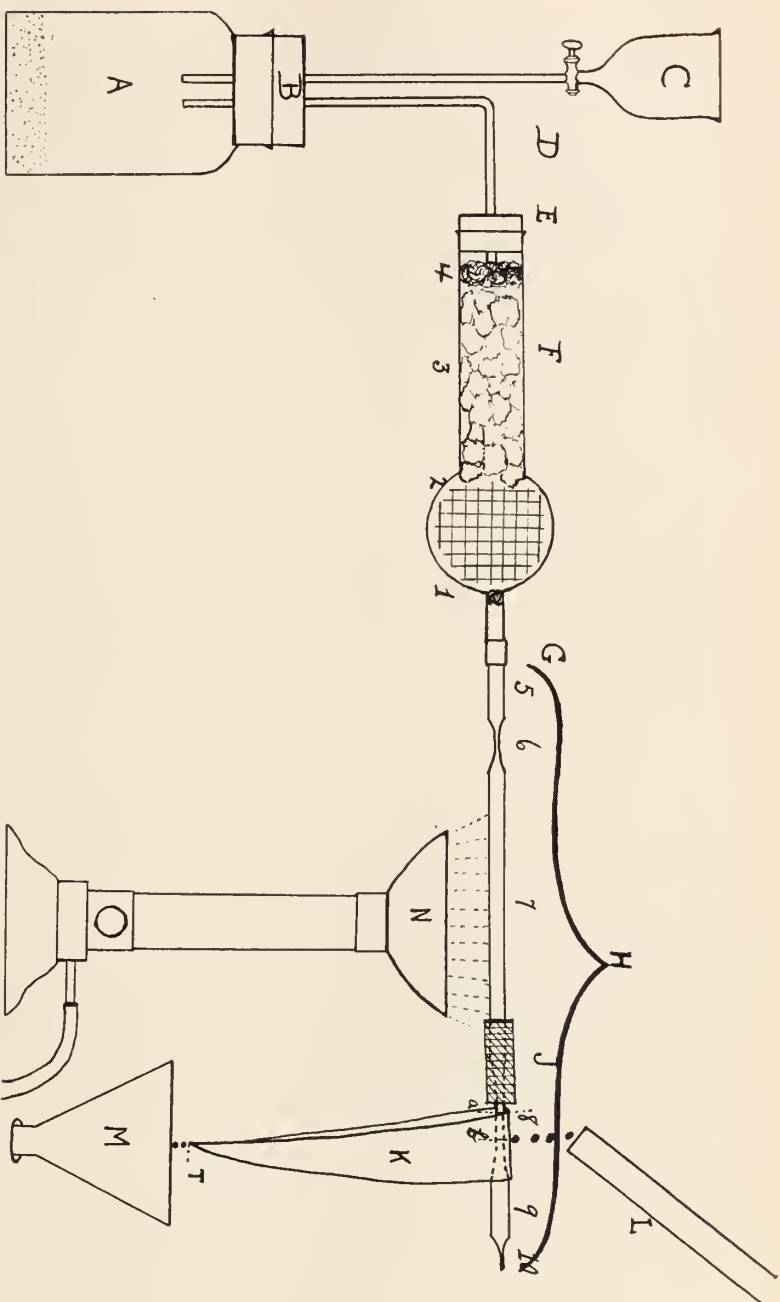
Fig. 1. The apparatus used in "marshing."

A = wide-mouthed bottle, capacity 100 to 200 c.c.
B = glass or rubber stopper.
C = dropping funnel with stop-cock.
D = delivery tube.
E = rubber stopper.
F = calcium chlorid tube containing, at 1, a glass wool plug; at 2, a filter paper or absorbent cotton saturated with lead acetate; at 3, neutral granulated calcium chlorid; and at 4, another glass plug.

G = rubber tubing connecting F to H.
H = glass depositing tube.
I = cold water conduit.
J = nickel gauze.
K = filter paper.
M = collecting funnel.

sulfuric acid and stirring) on a porcelain plate. The sulfuric acid solution free from all nitric acid and containing the arsenic is ready for "marshing."

The method used for "marshing" and preservation of the mirrors is a modification of the method used by Thomson.[30] The apparatus used consists of a wide-mouthed bottle (A) with a capacity of from 100 to 200 c.c., dependent on the amount of solution to be marshed, with a glass stopper (B)—vaselined— or a large rubber stopper (it may be a boiled-out rubber stopper) with double perforations containing a small dropping funnel (C) with a stop-cock, and an L-shaped delivery tube (D). The delivery tube (D) is inserted into a rubber stopper (E) which fits a small calcium chlorid tube (F) containing at 1, a glass wool plug; at 2, filter paper or absorbent cotton saturated with normal lead acetate of 1% solution and dry to remove hydrogen sulfid (this filter paper or cotton must be tested for lead acetate; when placed over the mouth of an ammonium sulfid bottle, it must turn black); at 3, neutral granulated calcium chlorid for drying the gas; and at 4, another glass wool plug. The calcium chlorid tube (F) is connected at its narrow end by means of a snugly fitting piece of rubber tubing (G) with the depositing tube (H)— a piece of hard glass tubing, medium-walled, from 6 to 7 mm. in external diameter, which will not collapse when heated to redness in the Bunsen flame. By means of a blast lamp the tube (H) is drawn down at 6 and 8—the constriction at 6 being made last, after measuring the internal dimension at 8 as directed hereafter—and drawn out to a tip at 10. The length of (H) is approximately 2 cm. at 5, 2 cm. at 6, 10½ cm. at 7, and 4½ cm. at 8. At 8 the tube is drawn down to about 3.0 to 2.9 mm. in external diameter and then measured internally by noting and marking with glass pencil the points at which two nails, 2.7 and 2.2 mm. in diameter measured by calipers, or wires are obstructed. The point of obstruction of the larger nail (a) is the site at which the deposition of the mirror is begun and is nearer the generator, while the point (b) at which the smaller nail is obstructed merely gives a gauge as to the range within which we can deposit the mirror so that there will not be too great a difference in the internal diameters in which the deposit occurs. The distance between the two points of obstruction should be about 1 cm. J is a small piece of nickel gauze (iron or copper gauze will serve tho somewhat unsatisfactory because easily melted or corroded) about 2 to 3 cm. long and wide enough to fit around the depositing tube (H) a little more than once. It is adjusted so that it will be about 2 mm. from the deposition line (a). K is a small piece of thin filter paper, lens paper, or tissue paper, about 2 to 3 cm. wide, cut so that folded it will hang over the tube (H) with its near edge on the line (a) and with two points meeting at (T), so that the cold water (10 to 20 C.) run onto it from above by means of the tube (L) connected to a cold water reservoir for the purpose of cooling the depositing tube (H) at 8 (the cooling is necessary to obtain a single and complete mirror), will run into the collecting funnel (M), which passes through a hole in the table and then into a large refuse bottle or drain pipe.

When the worker is ready for "marshing," about 20 grams of platinized granulated zinc (Baker and Adamson's platinized granulated zinc, Serial 2772, is satisfactory, sensitive and free from arsenic) are placed in the bottom of the generator (A), and about 10 to 15 c.c. of concentrated 1.84 stock sulfuric acid diluted with from 10 to 15 c.c. distilled water are run onto it through the funnel (C). This causes a violent evolution of hydrogen, which sweeps

[30] Chemical News, 1902, 86, p. 179; 1903, 88, p. 228; 1906, 44, p. 156.

all the air from the apparatus. In the meantime the solution to be "marshed" is placed in a small Erlenmeyer flask and brought to boil, removed from the flame, and the ferric iron reduced by the addition of a few crystals of fresh, chemically pure stannous chlorid (a rather large quantity will not inhibit the evolution of arsin, but a few crystals generally suffice) and shaken. Complete reduction occurs in about 5 minutes and is tested by adding a few drops of the solution to a solution of potassium sulfocyanate on a white porcelain plate. If reduction is not complete, the solution is warmed again, but not boiled, and more stannous chlorid added. When reduction is complete, the flask is stoppered and allowed to cool. The Marsh apparatus, free from air (tested by collecting the gas at 10 by upward displacement in a small test tube, and igniting) is tested for leaks by passing a small flame along its entire length, ignition occurring if leaks are present. The deposition tube is now freed from moisture by gently warming with a flame starting at 5 and passing to 10. The wing top Bunsen flame (N) is now lighted—blue flame— and placed so that it will heat the tube (H) red hot for about 7 cm. along 7, the flame touching the nickel gauze (J). Now the paper (K) is put into place and a small but fairly rapid stream of cold tap water is allowed to run over it. The evolution of hydrogen is gauged by the size of the hydrogen flame at 10, which must be about 1 to 2 cm. in length during the entire "marshing." When the violent evolution occasioned has subsided and the apparatus is ready, the unknown solution is poured into the dropping funnel (C), a few cubic centimeters at a time, and allowed to run into the generator. The evolution of hydrogen is regulated by adding a few cubic centimeters of distilled water to hasten, and stock sulfuric acid solution (equal parts of concentrated 1.84 sulfuric acid and distilled water) to retard it. (This was more satisfactory than the routine method of using certain amounts of three different concentrations of sulfuric acid.) The entire unknown solution should be run into the Marsh apparatus in about 10 to 20 minutes and "marshing" continued for 35 to 40 minutes after the last part of the unknown solution has been added. Tests have shown that practically all the arsin has passed quantitatively over by this time provided the generator A is not too large and the evolution of hydrogen is kept up. The flame (N) is then turned out and the tube (H) allowed to cool (keeping up the evolution of hydrogen). By means of a small blast flame the tube (H) is then first sealed at 10, the stop-cock of funnel (C) rapidly opened, and the tube finally sealed off at 6. The arsenic mirror thus sealed in hydrogen may be kept in the dark for long periods of time without deterioration. In the following analyses fractions of the unknown solution were taken which would give an arsenic mirror reading between 2 and 10 micromilligrams. The standard arsenic solution for making the control mirrors was prepared by dissolving 1.33 grams (or fraction thereof) of chemically pure arsenic trioxid in about 100 c.c. of boiling distilled water with the aid of sodium hydroxid or sodium carbonate added drop by drop until the crystals of arsenic trioxid have completely gone into solution, and diluting to one liter with cold, freshly boiled, distilled water. One cubic centimeter of this solution diluted to 100 c.c. gives the standard, one cubic centimeter of which equals 10 micromilligrams of arsenic. This diluted standard does not keep well, so that it must be freshly prepared at frequent intervals from the stronger solution."[31]

[31] Clark and Woodman: Circular 99, U. S. Department of Agriculture, Bureau of Chemistry.

THE DISTRIBUTION OF ARSENIC IN THE TISSUES OF TUBERCULOUS
ANIMALS GIVEN VARIOUS ARSENIC PREPARATIONS

EXPERIMENT 1.—A rabbit (2800 gm.) was inoculated with human tubercle bacilli in the right eye and 143 days later, when the entire bulb of the right eye was involved with tuberculosis, was given intravenously 6 mg. of arsenic in the form of sodium arsenite in 6 c.c. of water; 2 days later another 6 mg. arsenic were given, and 1 day later another 6 mg. of arsenic. Eight hours after the last injection the animal was bled to death and the organs were analyzed. The normal left eye contained 1 mmg. arsenic in 3 gm.; tuberculous right eye 2 mmg. in 2 gm.; blood 3 mmg. in 20 gm.; spleen 3 mmg. in 2 gm.; right kidney 10 mmg. in 7 gm.; left kidney 21 mmg. in 8 gm.; lungs 6 mmg. in 9 gm.; and the liver 60 mmg. in 12 gm.

EXPERIMENT 2.—A guinea-pig was inoculated in the left groin with 0.05 mg. human tubercle bacilli and 58 days later was given subcutaneously 0.5 c.c. of a 2% solution of sodium cacodylate in physiologic salt solution, and on succeeding days 0.75 c.c., 1.0 c.c., 1.25 c.c., and 2.0 c.c., respectively. The animal was killed by bleeding 8 hours after the last injection and the tissues were analyzed. The peribronchial glands contained 21.7 mmg. of arsenic in 3.0 gm.; spleen 15.8 mmg. in 2.5 gm.; testes 18.8 mmg. in 3.0 gm.; blood 87.5 mmg. in 17.5 gm.; lungs 17.8 mmg. in 5.0 gm.; kidneys 21.2 mmg. in 4.0 gm.; and liver 32.0 mmg. in 8.0 gm. The inguinal glands were large and caseous; the peribronchial gland was hard and enlarged. The spleen was enlarged and full of numerous small necrotic areas; the liver pale and necrotic; the lungs, kidneys, and testes normal.

EXPERIMENT 3.—A guinea-pig was inoculated in the left groin with 0.05 mg. human tubercle bacilli and 58 days later was given subcutaneously 1 c.c. of 1% atoxyl in physiologic salt solution, the second day 1 c.c., the third day 2.0 c.c., the fourth day 1.5 c.c. (animal sick and becoming emaciated), and the fifth day 1.0 c.c. Eight hours after the last injection the animal was bled to death and the tissues were analyzed. The local lymph glands (caseous) contained 50.0 mmg. arsenic in 3.0 gm.; retroperitoneal glands 24.2 mmg. in 2.5 gm.; peribronchial glands 15.6 mmg. in 1.5 gm.; blood 94.0 mmg. in 6.0 gm.; spleen 31.3 mmg. in 2.5 gm.; testes 14.7 mmg. in 3.0 gm.; and the liver 68.9 mmg. in 11.0 gm. Local, retroperitoneal, and peribronchial glands were enlarged. The spleen was full of miliary tubercles. There were many small necrotic areas in the liver. Peritoneal hyperemia was noted. The animal was emaciated.

EXPERIMENT 4.—A guinea-pig was inoculated in the left groin with 0.05 mg. human tubercle bacilli and 30 days later was given subcutaneously 4.5 c.c. of 1% arsacetin solution and the next day 8.0 c.c. Two hours after the last injection the animal was bled to death and the tissues were analyzed for arsenic. The local inguinal glands contained 60.0 mmg. of arsenic in 2 gm.; peribronchial glands 60 mmg. in 2 gm.; blood 750 mmg. in 17.0 gm.; lungs 300 mmg. in 5 gm.; spleen 60 mmg. in 3 gm.; kidneys 750 mmg. in 6 gm.; and the liver 225 mmg. in 13 gm. The local retroperitoneal, and peribronchial lymph glands were enlarged. There were small necrotic areas in the liver; the spleen was full of necrotic areas; the lungs also showed a few such areas. The rest of the organs appeared normal.

EXPERIMENT 5.—A guinea-pig was inoculated in the left groin with 0.05 mg. human tubercle bacilli and 30 days later was given subcutaneously 4.5 c.c. of 1% neosalvarsan and the next day another 4.5 c.c. One and one-half hours

after the last injection the animal was bled to death and the tissues were analyzed for arsenic. The local caseous inguinal glands contained 12 mmg. of arsenic in 3 gm.; retroperitoneal glands 5 mmg. in 1 gm.; spleen 12 mmg. in 2 gm.; blood 50 mmg. in 19 gm.; lungs 50 mmg. in 4 gm.; kidneys 50 mmg. in 5 gm.; and the liver 50 mmg. in 10 gm. The local and retroperitoneal glands were enlarged; peribronchial glands only slightly so. The liver and the spleen were full of small necrotic areas, and the lungs showed a few small necrotic areas. The rest of the organs appeared normal.

As a result of these experiments it is noted that:

Arsenic in simple crystalline salt form, as sodium arsenite, sodium cacodylate, atoxyl, arsacetin, and neosalvarsan, administered to tuberculous animals parenterally, is found in the liver, lungs, kidneys, blood, spleen, and tuberculous tissues (lymph glands of guinea-pigs and eye of rabbit), the concentrations in the various tissues not greatly differing. No evidence of accumulation in the tuberculous tissues was obtained.

Incidentally, since tin forms salts like arsenic, in which the tin is in the negative radical, the following experiments were performed to test whether sodium stannate was germicidal toward the tubercle bacillus.

A uniform suspension of human tubercle bacilli was added in equal amounts (5 drops) to 5 c.c. each of the following concentrations of sodium stannate: 0.001, 0.01, 0.1, and 1.0%, all in duplicate. At the same time 7 controls were made with 5 c.c. distilled water, and placed in the incubator at 37 C. for 48 hours (shaken at frequent intervals). At the end of this time the suspensions were injected subcutaneously into the left groin of normal guinea-pigs. It suffices to state that all the guinea-pigs receiving the stannate-treated bacilli developed a marked tuberculosis which did not differ materially from that of the controls. Sodium stannate, even in concentrations as high as 1.0% for 48 hours at 37 C., is therefore non-germicidal toward the human tubercle bacillus; no evidence even of attenuation was observed.

SUMMARY AND CONCLUSIONS

Sodium arsenite in dilution of from 0.1% to 0.0001% and sodium cacodylate in dilution of from 2% to 0.002% have no germicidal action on human tubercle bacilli in 24 hours at 37 C.

Mercury cacodylate in dilutions of from 1% to 0.001% has a germicidal action on human tubercle bacilli in 24 hours at 37 C. This action is in all probability due to the mercury and not to the cacodylate radical.

Atoxyl, arsacetin, and neosalvarsan in dilutions of from 1% to 0.001% have no germicidal action on human tubercle bacilli in 24 hours at 37 C.

These compounds, representing the commonly used inorganic and organic preparations of arsenic, cannot be said to have any specific action on human tubercle bacilli, and if of value in the treatment of tuberculosis, are so only because of their favorable influence on metabolism. That tissue compounds would produce combinations with arsenic in the animal body which might be tuberculocidal is very unlikely, for a review of the clinical literature presents no evidence of any specific action of arsenic compounds in tuberculosis.

Arsenic in simple crystalline salt form—sodium arsenite, sodium cacodylate, atoxyl, arsacetin, and neosalvarsan—administered to tuberculous animals parenterally is found in the liver, lungs, kidneys, blood, spleen, and tuberculous tissues (lymph glands of guinea-pigs and eye of rabbit), the concentrations in all these tissues not greatly differing. No evidence of accumulation in the tuberculous tissues was obtained.

Sodium stannate, even in concentration as high as 1% for 48 hours at 37 C., is not germicidal toward human tubercle bacilli.

A CASE OF INFECTION OF LYMPH GLANDS WITH BACILLUS PARATYPHOSUS B *

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The patient, a private in the United States Army, presented symptoms suggestive of Hodgkin's disease, and his case was at first so diagnosed and reported.

In March, 1914, the patient noticed, on a march, that the left leg was becoming "numb" and swollen. Two months later the glands in the left side of the neck were enlarged. He had not suffered any pain. On admission to hospital, June 21, 1914, he still complained of swelling and numbness in the left leg and thigh.

The general physical condition and nutrition of the patient were good. He was 70½ inches in height, and 139 pounds in weight. His skin was relaxed and showed no pigmentation. The mucous membranes were pale. Eyes, ears, and nose showed no significant abnormalities; the teeth were fairly good, but poorly cared for.

The cervical glands on the right side, both superficial and deep, were hard and slightly movable. There was a slight infiltration of the surrounding tissue. The skin was not adherent to the glands. On the left side of the neck there was a large mass, hard, discrete, and movable. Pain developed in the glands on the left side of the neck, where there was some redness of the skin. Incision was made and pus drained.

Aside from increased resonance in the left infra-clavicular region and a few moist râles in the left side of the chest, the lungs and chest were normal and the sputum negative.

The heart and blood vessels were normal. The systolic blood pressure was 135; the diastolic, 70; pulse pressure, 65; pulse rate, 85. There was a slight dilatation of the superficial veins of the abdomen.

In the left lower abdomen there was a large, hard, and slightly movable mass. The left internal iliac glands were enlarged, hard, and movable.

The left leg at the thigh was 1½ inches larger than the right; at the middle of the calf, 1 inch larger than the right. Edematous swelling was present, as a result probably of pressure by the enlarged iliac glands on the left iliac vessels. The axillary glands on both sides were enlarged, hard, and movable. There was no evidence of obstruction in the upper extremities.

The feces were negative. The urine showed a specific gravity of 1.008; hyaline and granular casts, and epithelial cells, squamous and round; genitourinary system otherwise normal. The blood showed 4,300,000 red cells, 10,400 white cells, hemoglobin 73%, small mononuclears 46.75%, large mononuclears

14.75%, transitional 0.50, neutrophiles 37.75%, mastcells 0.25%. Wassermann and luetin tests negative.

Temperature varied from 97.2 to 104.4.

In October, 1914, the patient began to complain of increasing pain in the left shoulder and the left arm. There were marked sensitiveness and redness over a mass on the left infra-clavicular space. A movable gland was excised and examined. Macroscopically there were evident small white nodes throughout the tissue. Sections revealed a uniform distribution of small round cells, regular in size, with also a few large cells. In some areas the cells were more closely crowded without increase of connective tissue. In a few small areas the cells were arranged in well-separated columns bounded by a connective tissue framework; these cells had no regular order and no apparent relation to the other cells. There was a distinct capsule limiting the gland.

From the pus, removed with a syringe from the suppurating gland in the neck, a short motile gram-negative bacillus was isolated. Cultures of this organism in agar yielded bluish-white colonies after 24 hours; in Loeffler's blood serum, a white distinct growth, with, at the end of 72 hours, a black line at water of condensation; blood agar gave a white growth which was more profuse than that on any other media. Cultures in Dunham's solution showed general cloudiness, slightly increased after 48 hours; no indol was produced. Nitrates were not reduced in 90 hours. In litmus milk the organism caused slight acidity and gave rise to a blue-green cream ring after 24 hours; after 48 hours the acidity had been increased, and the green cream ring was marked; at the end of 72 hours changes to white were discernible at the butt. On potato the bacillus gave a moist white growth after 24 hours, less moist at the end of 72 hours. In Russell's double sugar there was an acid butt, blue stem, and formation of gas at the end of 24 hours; after 48 hours, the blue color was marked and gas-formation still evident; and after 72 hours the medium was entirely blue except for the decolorized butt.

Table 1 shows the fermentative reactions of the bacillus on various sugars.

FERMENTATIVE REACTIONS OF THE BACILLUS ISOLATED ON VARIOUS SUGARS

Media	Fermentative Reaction After			
	24 Hr.	48 Hr.	72 Hr.	72 Hr.
Glucose............	40 %	40 %	42.5%	Acid
Levulose............	42.5%	45 %	47.5%	Acid
Galactose...........	45 %	45 %	45 %	Acid
Mannite............	60 %	57.5%	55 %	Acid
Maltose.............	25 %	45 %	50 %	Acid
Lactose.............	No gas	No gas	No gas	Alkaline
Saccharose..........	No gas	No gas	No gas	Alkaline
Dextrose............	Bubble; no gas	No change	No change	Alkaline
Starch..............	No change	No change	No change	Alkaline
Dulcite.............	70%	82.5%	85%	Acid
Raffinose...........	No gas	No gas	No gas	Alkaline

The organism failed to liquefy gelatin in 5 days.

The organism was agglutinated by B. paratyphosus B immune serum in dilution of 1:640. A known paratyphoid bacillus, used as control, was agglutinated by the immune serum in dilution of 1:5120. The blood serum of the patient failed to agglutinate the organism in dilutions of from 1:20 to 1:5120,

and failed to agglutinate the known paratyphoid bacillus in all dilutions. Sub-cultures of the organism were further identified as B. paratyphosus B in the laboratory of the Army Medical School at Washington, D. C.

INOCULATION EXPERIMENT

The pus was injected subcutaneously into the abdomen of a guinea-pig. An extensive area of induration followed, gradually extending under the skin of the abdomen and the thorax to dimensions of 1 by 2½ inches. From the clear serous exudate, drawn with a syringe, an organism was isolated in pure culture, corresponding in all its charac-teristics to B. paratyphosus B.

Thirteen days after inoculation the guinea-pig died. The indurated area was one-fourth inch thick, friable, yellow-white in color. No pus was present. The vessels of the abdomen, chest wall, and mesentery were congested. The lymph nodes of the intestine and the mesenteric glands were enlarged and congested. The spleen was markedly enlarged.

In the microscopic examination there was revealed an infiltration of the tissue with small round cells that almost completely destroyed the normal structures. Some of the blood vessels in the indurated tissue remained patulous and some of the muscle bundles were surrounded by varying areas of inflammatory product. The infiltrate toward the margin of the indurated area lay largely in the areolar tissue below the superficial muscular layers, and extended between the muscle bundles by way of the epimysium. The cells in the indurated area varied from large with abundant cytoplasm and distinct nuclei to small with small nuclei and little or no cytoplasm.

The spleen showed marked degeneration. There were areas irregularly placed showing no structure, the intervening areas being filled with large and small cells, like those in the inflamed tissue at the site of the injection. In these cells the nuclei were irregular as to size and shape, some apparently budding forms being seen. Especially notable were numerous cells with abundant cytoplasm and small round deeply staining nuclei; and other cells with much cytoplasm and large poorly staining nuclei.

Numerous enlarged glands were removed from the mesentery, one, after hardening, measuring 4 by 2 mm. The connective tissue in this gland stained poorly. The cellular elements appeared closely approximated with little con-nective tissue support. The regularity of the cells and their nuclei were marked throughout the gland. There were a few large cells with nuclei, but no diminutive or degenerated cells. There were numerous large lacunae filled with a poorly staining homogeneous material, with a slight crowding of cells around the lacunar periphery.

In the liver the vessels around the central veins were greatly distended and congested. Around the larger veins were areas of cell-destruction, show-ing the presence of foreign cells.

In the kidney there was cloudy swelling of the cells of the tubules with partial occlusion of the lumen; the cells remained in situ against the basement membrane and no inflammatory localizations or abscesses were found. Cells of the glomeruli showed cloudy swelling. A few blood vessels were dilated with blood elements. The proportion of mononuclear white cells as compared with that of polymorphonuclears was high.

Subcultures of each of the organisms isolated from the guinea-pig lesion and heart blood were identified as cultures of B. paratyphosus B at the laboratory of the Army Medical School at Washington, D. C.

There were no agglutinins present in the blood of the patient for either a known culture of B. paratyphosus B or the organism isolated. After treatment with an antogenous vaccine from April 20 to May 4, 1915, the patient clinically showed some improvement at the time of his discharge, on May 19, but there were still no agglutinins present in the patient's blood when last taken (May 10).

Cultures from blood, urine, and feces were negative for the presence of B. paratyphosus B.

Subsequent to the identification of the organism efforts were made to elicit a history of typhoid or any febrile condition at any previous time in the patient's life; but no such history could be obtained.

During a long period of observation the organism produced suppuration in one group of glands on two occasions, more than a month after surgical intervention; other greatly enlarged glands did not suppurate. Interference with the blood supply to still other glands or parts of glands appears to have aided suppuration. On inoculation of an animal with pus from the broken-down glands, death resulted before local necrosis had progressed to the stage of pus-formation, while but slight evidence of such existed in the tissues.

This case of multiple lymphadenitis may be added to the list of localized infections by this type of organism which manifest themselves as primary infections. These, as given by Park and Williams, are as follows: "Pyelitis (sometimes associated with septicemia), cystitis, arthritis, periostitis, phlebitis, cholecystitis, etc. It occasionally occurs as a complicating infection of other diseases."

CONCLUSION

The condition of multiple lymphadenitis described in this patient seems to have been caused by an infection with Bacillus paratyphosus B, and, so far as could be ascertained, the disease developed without a preliminary manifestation of a typhoid-like, gastro-enteric, or cholera-like type of infection.

SIMULTANEOUS INJECTIONS OF STREPTOCOCCI AND DAHLIA IN THE GUINEA-PIG *

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The effect of anilin dyes on micro-organisms in vitro has been investigated by a number of workers, among whom may be mentioned Rozsahegyi,[1] Stilling,[2] Drigalski and Conradi,[3] Simon and Wood,[4] and Churchman.[5] A review of the literature up to 1914 occurs in the first article by Simon and Wood. Since that time, reports by Shiga,[6] Hall and Taber,[7] and a second communication by Simon and Wood have appeared. The latter found that certain dyes in dilutions of 1 : 100,000, regardless of their color, may be capable of inhibiting the growth of certain bacteria. One of the dyes investigated, dahlia (a mixture of methyl violet and fuchsin), was found effective in inhibiting the growth of certain strains of streptococci when it was present in culture media in a concentration of 1 : 100,000.

Later, Ruhräh[8] used dahlia in cases of tonsillitis and in various skin infections. Several cases of extensive furunculosis of the scalp in this hospital were treated locally with dahlia solution with apparently good results in one case. The possibility then occurred to us of intravenous injections of the dye in systemic streptococcus infections.

First, we determined the minimal lethal dose of a 1 : 1000 solution of dahlia in normal salt solution to be 5 c.c. for a guinea-pig of 200 gm. when injected intravenously. When the dahlia was given in doses of from one-eighth to one-half of the minimal lethal dose, the animal showed practically no ill effects, with the possible exception of an occasional slight loss in weight. In determining the effect of the intravenous injections of dahlia on streptococcus infections, we never have used more than the sublethal quantities of dahlia mentioned. Further control animals which were injected intraperitoneally with sterile

[1] Centralbl. f. Bakteriol., I. O., 1887, 11, p. 418.
[2] Anilin Farbstoffe als Antiseptika und ihre Anwendung in der Praxis, 1890.
[3] Ztschr. f. Hyg. u. Infektionskrankh., 1912, 39, p. 283.
[4] Am. Jour. Med. Sc., 1914, 142, p. 247; p. 524.
[5] Jour. Exper. Med., 1912, 16, pp. 221, 822; 1913, 17, p. 373.
[6] Ztschr. f. Immunitätsf., 1914, 18, p. 65.
[7] Jour. Infect. Dis., 1914, 15, p. 566.
[8] Am. Jour. Med. Sc., 1915, 149, p. 661.

broth, instead of a 24-hour broth culture of streptococci, showed no effects of broth injections.

We used various strains of hemolytic streptococci, isolated from scarlet-fever sore throats. The strains used, with the exception of D, grew in dahlia broth, 1 : 100,000 concentration. Strain D did not grow in this dilution, but showed scant growth in dahlia broth, 1 : 200,000 concentration. Churchman, working with gentian violet, and Simon and Wood, working with dahlia, etc., have shown that the dyes do not affect all strains of streptococci alike. Measured quantities (0.3 to 1.5 c.c.) of 24-hour broth cultures of streptococci were injected intraperitoneally before or soon after an intravenous injection of the 1 : 1000 dahlia solution. Duplicate sets of animals were frequently injected.

Early in our work we obtained an unexpected result: control animals receiving streptococci survived without presenting any appreciable signs of illness, while the other animals, receiving the same amount of broth culture intraperitoneally and an intravenous injection of dahlia much below the fatal dose, died within 24 hours. That this did not remain an isolated occurrence is seen from Table 1.

Unfortunately, it was not always possible to work with animals of the same weight, and this difference may have influenced the result. For instance, in Experiment 7, the one control animal which died weighed one-fifth less than the heavier animal which was killed by the combined injections. Nevertheless, the fact stands out clearly that in the combined injections the dahlia acts detrimentally. Experiment 8 is of special interest because the animal weighing 200 gm. received in the 2.5 c.c. of dahlia solution an amount of dahlia corresponding to 1 : 80,000 of the body weight. This same streptococcus failed to grow in dahlia broth, 1 : 100,000. The concentration of the dye in the animal (its total weight being taken into consideration) was greater than that necessary to inhibit the growth of the streptococcus in vitro. In one instance only did it happen that, in a set of 4 animals, 2 receiving streptococci died, while only 1 of 2 receiving dahlia and streptococci succumbed. In another set of experiments 1 animal of each group died and 1 survived. None of the animals receiving Strain C died; on the other hand, some streptococci were so virulent that all the animals succumbed.

In an attempt to investigate the failure of dahlia to act beneficially in streptococcus infections, even when the strain was killed by the dye in vitro, some experiments were made testing the effect of serum as the culture medium. Blood was withdrawn from the hearts of guinea-pigs,

allowed to coagulate, centrifugated, and the serum collected under aseptic conditions. To measured quantities of the serum thus obtained concentrated solutions of dahlia were added in various proportions.

TABLE 1

RESULTS OF SIMULTANEOUS INJECTIONS OF STREPTOCOCCI AND DAHLIA INTO GUINEA-PIGS

Experiment	Weight of Guinea-pig in Grams	Strain of Streptococcus	Dosage	Result
1	200	A	1 c.c. 24 hour broth culture	Survived
	200	A	The same with 1 c.c. dahlia	Death in 16 hours
2	250	A	1 c.c. 24 hour broth culture	Survived
	230	A	The same with 1 c.c. dahlia	Death in 70 hours
	190	A	The same with 1 c.c. dahlia	Death in 70 hours
3	405	B	2 c.c. 24 hour broth culture	Survived
	350	B	1.5 c.c. of 24 hour broth culture with 1.5 c.c. dahlia	Survived
	315	B	1.5 c.c. of 24 hour broth culture with 1.5 c.c. dahlia	Continuous loss of weight until death on 8th day. Streptococcus recovered from heart
4	410	B	2 c.c. 24 hour broth culture	Survived
	380	B	The same with 1 c.c. dahlia	Death in 36 hours. Streptococcus recovered from heart
5	310	B	2 c.c. 24 hour broth culture	Survived
	400	B	The same	Survived
	380	B	The same with 1.5 c.c. dahlia	Continuous loss of weight until death on 7th day
	370	B	The same with 1 c.c. dahlia	Survived
	320	B	The same with 1 c.c. dahlia	Death in 60 hours. Streptococcus recovered from heart
6	540	B	2 c.c. 24 hour broth culture	Survived
	580	B	The same with 1 c.c. dahlia	Continuous loss of weight until death on 14th day
7	215	D	0.5 c.c. 24 hour broth culture	Death in 33 hours. Streptococcus recovered from heart
	300	D	The same	Survived
	225	D	The same with 2 c.c. dahlia	Death in 18 hours. Streptococcus recovered from heart
	270	D	The same with 2 c.c. dahlia	Death in 48 hours. Streptococcus recovered from heart
8	195	D	0.5 c.c. 24 hour broth culture	Survived
	230	D	The same	Survived
	200	D	The same with 2.5 c.c. dahlia	Death in 18 hours. Streptococcus recovered from heart
	235	D	The same with 2.5 c.c. dahlia	Survived
9	230	D	1 c.c. 24 hour broth culture	Survived
	270	D	The same	Death in 23 hours. Streptococcus recovered from heart
	240	D	The same with 2.5 c.c. dahlia	Death in 18 hours. Streptococcus recovered from heart
	250	D	The same with 2.5 c.c. dahlia	Death in 19 hours. Streptococcus recovered from heart

The tubes were then inoculated with Strain D (controlled anew). The cocci failed to grow in dahlia broth, 1 : 100,000, but there was an occasional scant growth in dahlia broth, 1 : 200,000. In the presence

of serum a concentration of the dye of 1 : 10,000 was necessary to inhibit growth, while a concentration of 1 : 20,000 was not effective. The dye therefore fails to fulfil the postulate made by Wright:[9] "that a bactericidal drug in order to have chemo-therapeutic effect must not only be capable of killing the micro-organisms in vitro, but also must not combine with any of the chemical constituents of the normal body, otherwise that portion will be rendered inert towards the bacteria." These results explain why dahlia, under the conditions of our experiments, fails to act beneficially, but they do not explain the injurious action of the combined injections.

DISCUSSION

Our experiments show that a substance capable, in high dilutions of broth cultures, of destroying streptococci, when injected intravenously may not only fail to have a beneficial effect on experimental streptococcal infections, but, on the contrary, may act injuriously. It is not unlikely that the virulence of the different strains enters into consideration. Thus, it may happen that neither the controls nor the animals receiving the combined injections are affected in any way. The other extreme is that all animals succumb with or without dahlia. Between these two extremes we have strains of such degree of virulence that the animals receiving the streptococci alone, survive, while those receiving the combined injections die. We incline to the assumption that the dye renders the organism less resistant to the attack of the streptococci—in the sense suggested by Ehrlich, combining with the elements of the living body and thus interfering with their physiologic function.

The question naturally arose whether the dahlia would injure some of the defensive mechanisms of the organism. Therefore, one of us (Sauer) tested the influence of dahlia on the opsonic power of the guinea-pig serum as well as the possibility of an influence on the complement of the blood. In the opsonic experiments guinea-pig leukocytes and guinea-pig serum were used together with streptococci or staphylococci. The dahlia was added in a total concentration of 1 : 4000. In the control experiments the dahlia salt solution was replaced by physiologic salt solution. In 10 series of experiments no influence of the dahlia on the opsonic index could be observed.

On the addition of dahlia, in total concentrations varying between 1 : 400,000 and 1 : 2000, to a hemolytic system there was no influence

[9] Lancet, 1912, 183, pp. 1633, 1701.

whatever on the reaction. Neither did the serum of a guinea-pig (300 gm.) that had received 3 c.c. of a dahlia solution, 1 : 1000, intravenously 15 minutes previously, behave differently from the serum obtained before the dahlia injection.

In conclusion, it seems evident from these experiments that intravenous injection of dahlia in general streptococcal infections is not warranted as a therapeutic measure.

THE CORRELATION OF THE VOGES-PROSKAUER AND METHYL-RED REACTIONS IN THE COLON-AEROGENES GROUP OF BACTERIA *

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Bacillus coli was first isolated by Escherich, from the feces of a cholera patient in 1884. It was soon recognized as a normal inhabitant of the intestinal tract of man and other animals, and for the past two decades the colon-aerogenes group of bacteria has attracted considerable attention from bacteriologists; particularly those interested in sanitation. Numerous attempts have been made to differentiate colon-like bacteria from different sources by their cultural characteristics, by agglutination reactions, complement-fixation tests, pathogenicity, etc., but until very recently the results were so uniformly negative that the entire group of lactose-fermenting bacteria is held, by some, to be characteristically of intestinal origin. In routine water and milk analyses the presence of colon-like organisms is regarded, by many, as an index of pollution. There is considerable evidence accumulating, however, to show that the animal intestine is not the sole and exclusive habitat of colon-like bacteria. Prescott,[1] in this country, and Papasotiriu,[2] in Germany, report the presence of colon-like organisms on grains of various kinds. Prescott regards those from grain and feces as identical. He believes that multiplication actually takes place on the grain and that their presence cannot be explained by contamination from the air and dust. Rogers and his co-workers[3] of the U. S. Department of Agriculture have recently isolated 166 specimens of colon-like bacteria from 33 samples of dry grain (corn, barley, wheat, and oats). They also obtained 14 cultures from 2 samples of green oats, which had been opened aseptically, and are therefore inclined to believe that these were growing in the milk of the immature grain.

In view of the fact that organisms which are apparently B. coli are not uncommon on grains, their presence in water and milk cannot be regarded as prima facia evidence of fecal contamination. Some

[1] The Biological Studies of the Pupils of Wm. T. Sedgwick, 1906, p. 208.
[2] Arch. f. Hyg., 1901, 41, p. 204.
[3] Jour. Infect. Dis., 1915, 17, p. 137.

authorities, particularly among the Germans, are inclined to disregard the colon test entirely, on account of the wide distribution of the organisms in nature. Prescott and Winslow[4] point out that in the interpretation of the colon test, the number and not the mere presence of B. coli should be taken as an index of pollution.

Rogers and his colleagues[3] have succeeded in differentiating colon-like organisms occurring on grain from those of fecal origin by the gas ratio. They found that strains from bovine feces were characterized by the fermentation of dextrose with the formation of hydrogen and carbon dioxid in about equal volumes, while those occurring on grains produce two or more times as much carbon dioxid as hydrogen. The accurately determined gas ratio is not practical for routine work, but, if some simple and rapid test could be found which would be well correlated with the gas ratio, the colon test would be more reliable and valuable as a criterion of fecal content than it is at present.

Clark and Lubs,[5] also of the U. S. Department of Agriculture, point out that there is absolute correlation between the gas ratio and the hydrogen-ion concentration. The bovine fecal strains are characterized by a relatively high hydrogen-ion concentration, those from grain by a low hydrogen-ion concentration. The difference in H^+ ion concentration between these two groups is such as to be easily recognized by appropriate indicators. With methyl red, for example, the high-ratio group is yellow, or alkaline; while the low-ratio group is red, or acid.

It is the purpose of this paper to record certain observations on the indicator test suggested by Clark and Lubs, and to point out the correlation between this test and certain other cultural characters as determined from a study of 167 organisms obtained from various sources.

Source of Cultures

The collection of organisms studied contained 19 cultures from the feces of 8 horses, 31 from 10 pigs, 20 from 6 cows, 22 from 8 sheep, 19 from 6 samples of raw sewage, 20 from 6 samples of septic sewage, and 25 from 7 samples of human feces. Eleven cultures received from the collection of the American Museum of Natural History were also studied. They contained 2 organisms marked B. coli communis (A and B), 4 of B. communior (A_1, A_2, B, and C), 2 of B. acidi lactici (A_2 and B), and 1 each of B. aerogenes, B. cloacae, and B. mucosus capsulatus.

Method of Isolation

The samples from sewage were plated on litmus lactose agar, and after incubation at 37 C. for 24 hours, 3 or 4 acid colonies were fished into lactose

[4] Elements of Water Bacteriology, 1915, p. 149.
[5] Jour. Infect. Dis., 1915, 17, p. 160.

broth, which, in turn, was incubated at the body temperature. If gas was produced in 24 or 48 hours, new litmus-lactose-agar plates were made and typical, well-isolated colonies were fished onto agar slants.

The samples from animals were collected in sterilized tumblers, or sample bottles, immediately after evacuation. A small amount of the interior of the solid material was suspended in sterile physiologic salt solution. This suspension was then plated on litmus lactose agar and otherwise treated in exactly the same manner as the samples from sewage.

<div align="center">CHARACTERS STUDIED</div>

The organisms were studied for morphology; staining by Gram's method; ability to produce acid and to clot milk; liquefaction of gelatin; fermentation of various carbohydrates; the Voges-Proskauer reaction; and the H^+-ion concentration as determined by the reaction to methyl red and paranitrophenol.

Morphology.—With very few exceptions all the organisms were short rods. From one sample of sheep feces, 4 cultures which were distinctly larger were obtained, but such variations in size as were observed among the different cultures were not correlated with any other characters.

Gram Stain.—All the organisms studied gave a negative Gram test. The stain was made by treating smears 1 minute with anilin oil gentian violet, then 1 minute with Gram's iodin solution, after which they were immersed in alcohol for 5 minutes, washed in water, and stained with dilute safranin for 15 seconds. All the cultures stained pink. Streptococcus lacticus and young cultures of B. subtilis similarly treated, as a check, were distinctly positive.

Milk.—All the organisms produced acid and clotted litmus milk in 48 hours at the body temperature.

Gelatin.—Gelatin was not liquefied by any of the organisms in this collection. The test was made at 20 C. and observed for 20 days.

Carbohydrates.—Tests for the quantity of acid and gas-production were made in dextrose, galactose, lactose, maltose, saccharose, raffinose, mannite, glycerin, dulcite, salicin, and inulin.

Voges-Proskauer Reaction.—In the first series of Voges-Proskauer tests, 5 c.c. of a 10% KOH solution were added to 3 to 4 c.c. of a 48-hour dextrose-broth culture in each of 2 test tubes. One tube was allowed to stand at room temperature, while the other was boiled as recommended by West[6] and Rivas.[6] It was found that the boiling produced a yellow color which obscured the eosin-like color formed when the Voges-Proskauer test was positive. In later tests I employed the peptone dextrose di-potassium hydrogen phosphate medium suggested by Clark and Lubs[5] for use in the determination of hydrogen-ion concentration. This medium was almost colorless, and I found that by adding 5 c.c. of a 10% KOH solution to about 4 c.c. of the culture and incubating over night a very distinct test was procured.

Indicator Tests.—The medium employed for these tests consisted of 0.5% di-potassium hydrogen phosphate; 0.5% Witte's peptone; and 0.5% dextrose, sterilized in the Arnold. Two series of experiments were conducted; at 37 and 30 C., respectively.

The technic in one of the two sets was somewhat different from that employed in the other. In the body-temperature series, flasks containing

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[6] Cited by West, Am. Jour. Pub. Hyg., 1909, 19, p. 227.

100 c.c. of media were used. At intervals of 2, 3, 4, and 6 days, two 5-c.c. portions were removed. To one portion was added 0.2 c.c. of paranitrophenol and to the other 0.1 c.c. of methyl red.

In the series at 30 C., each organism was inoculated into 4 test tubes containing 10 c.c. each of media. After 2 days 5 c.c. from one of the tubes were tested with methyl red, and to the remainder of the culture in the test tube were added 5 c.c. of a 10% KOH solution for the Voges-Proskauer reaction. A 2nd and a 3rd tube were treated in the same manner after 3 and 5 days' incubation, respectively. The contents of the 4th tube were tested on the 7th day with methyl red and paranitrophenol.

DISTRIBUTION OF ORGANISMS

That the group of organisms studied was a fairly representative one is seen from their distribution among the 4 main groups of MacConkey,[7] as shown in Table 1.

TABLE 1

Distribution of Colon-like Organisms From Various Sources Among MacConkey's Groups

Source	B. [acidi] lactici Saccharose — Dulcite —	B. coli [communis] Saccharose — Dulcite +	B. coli [communior] Saccharose + Dulcite +	B. [lactis] aerogenes Saccharose + Dulcite —	Total
Horse...............	3	1	12	3	19
Pig.................	14	7	6	4	31
Cow................	5	5	5	5	20
Sheep..............	0	1	10	11	22
Raw sewage........	5	6	4	4	19
Septic sewage......	9	2	4	5	20
Man...............	18	4	1	2	25
Totals.........	54	26	42	34	156

TABLE 2

Correlation between the Source of B. coli and Saccharose-Fermentation

Source	Saccharose +		Saccharose —	
	Number of Organisms	Percentage	Number of Organisms	Percentage
Horse...................................	15	79	4	21
Cow....................................	10	50	10	50
Sheep..................................	21	95	1	.05
Man....................................	3	12	22	88
Pig....................................	10	32	21	68

Fifty-four cultures are in the B. [acidi] lactici group (saccharose-negative and dulcite-negative); 26 are B. coli [communis] (saccharose-negative, dulcite-positive); 42 are in the B. coli [communior] group (saccharose-positive and dulcite-positive); and 34 fall in the B. [lactis] aerogenes group (saccharose-positive, dulcite-negative).

[7] Jour. Hyg., 1905, 5, p. 333; 1909, p. 86.

If the organisms are classified by their ability to ferment saccharose alone, as suggested by Smith[8] and Howe,[9] there is a more marked correlation with the source than is observed in the MacConkey grouping.

Of the organisms from the horse 79%, and of those from the sheep 95% fermented saccharose, while only 32% of those from pigs and 12% of those from man were saccharose-positive. In a study of lactose-fermenting bacilli present in soil, a preliminary test on 33 organisms which produced gas from lactose, showed that 32, or 79%, also formed gas in saccharose.

THE INDICATOR TEST

The Indicator.—Clark and Lubs[5] recommend a temperature of 30 C., an incubation period of from 3 to 5 days, and methyl red as the indicator. In this study it was found that there is little choice between methyl red and paranitrophenol. With the former, the neutral tints were easier to detect, so that in a quick laboratory test results recorded neutral with methyl red might be regarded as acid to paranitrophenol unless standards of comparison were at hand. After a preliminary trial of both indicators, methyl red was selected for the routine work.

The Effect of Temperature.—The practice in routine water and milk analyses has always been to incubate at room or body temperature. For B. coli 37 C. has been most commonly employed, and in the 1912 edition of the Standard Methods for Water Analysis of the American Public Health Association 40 C. is recommended. Substitution of 30 C. for the indicator test would be warranted only if the results obtained at the lower temperature are markedly different from those at body heat. Clark and Lubs[5] do not give any comparative data, and in the study of 167 strains recorded here, the reaction to methyl red was not influenced appreciably by the temperature of incubation. A comparison of the results after 5 days' incubation gave 1 culture acid at 37 C. and neutral at 30 C.; 5 neutral at 37 C. and acid at 30 C.; 4 neutral, 12 alkaline, and 145 acid at both temperatures.

The Effect of Period of Incubation and Temperature.—As to the effect of time of incubation and temperature combined on the reaction, it was found that one organism was neutral at both temperatures until the 3rd day, after which it was acid. Two cultures were constantly acid at 30 C., while at 37 C. the reaction changed from neutral on the 2nd day to acid on the 3rd and subsequent days. In these two instances, the final reaction was reached sooner at the lower tempera-

[8] Centralbl. f. Bacteriol., 1895, 18, p. 494.
[9] Science, 1912, 35, p. 225.

ture. The reverse was the case in 11 other cultures, which were acid after 2 days at the body temperature, but neutral at 30 C. until the 3rd day, after which they were also acid. Three cultures which were acid at both temperatures on the 2nd day showed signs of reversion at the lower temperature, but not at body temperature. One of these began to revert on the 3rd day but was not distinctly alkaline until the 7th day. The other two showed no signs of reversion until the 5th day. This reversion is an important phenomenon and should be investigated further. Two cultures neutral at 30 C. after 2 days, were alkaline after 3 days; while at the body temperature they were alkaline in 2 days. In all other cultures (158), there was no change in the reaction at either temperature after 48 hours. The evidence as to the effect of the temperature and the incubation period on the reaction with methyl red is summarized in Table 3, which indicates that the final reaction is reached more quickly at the body temperature. With an incubation period of from 3 to 5 days, there is little choice between 37 C., and 30 C.; but if the time of incubation is reduced to 48 hours, the body temperature seems preferable.

TABLE 3

Effect of the Temperature and the Period of Incubation on the Reaction with Methyl Red

Reaction	Incubation at 37 C. in Days				Incubation at 30. C. in Days			
	2nd	3rd	4th	6th	2nd	3rd	5th	7th
Acid.....................	143*	146	146	146	139	149	148	148
Neutral..................	12	9	9	9	18	6	7	6
Alkaline.................	12	12	12	12	10	12	12	13

* The figures indicate the number of cultures.

CORRELATION BETWEEN THE VOGES-PROSKAUER AND METHYL-RED REACTIONS

The 12 organisms which were alkaline to methyl red gave the Voges-Proskauer reaction, while all cultures that were acid or neutral failed to give this reaction. It is interesting that the culture which at 30 C. became alkaline on the 7th day of incubation, also gave a posiitve Voges-Proskauer reaction on that day but not earlier. With few exceptions the neutral tints were only slightly removed from a distinct acid reaction, but neutrality did not seem to be correlated with source or any other character.

The first series of Voges-Proskauer tests was made by Mr. N. C. Pervier of the chemistry department. Beef-extract dextrose broth was incubated at the body temperature for 48 hours. The second series was

made about 3 months later by Mrs. S. B. More, also of the chemistry department. She had no previous knowledge of the cultures or of their reactions. The dextrose peptone di-potassium hydrogen phosphate medium of Clark and Lubs[5] was incubated at 30 C. The results obtained by the two workers were absolutely confirmatory. From the small number of organisms in this collection giving the Voges-Proskauer reaction it seems that this reaction (the production of acetyl-methyl-carbinol from dextrose) is a constant physiologic character. The constancy of this reaction should be further investigated.

An interesting relation exists between the source, and the Voges-Proskauer, and methyl-red reactions. From Table 4 it is seen that all organisms which were alkaline to methyl red and which gave a positive Voges-Proskauer reaction had been obtained from sewage. Of 39 cul-

TABLE 4

Correlation of the Voges-Proskauer and Methyl-red Reactions with the Source

Source	Reaction with Methyl Red— 7 Days' Incubation at 30 C.		Voges-Proskauer Reaction	
	Acid or Neutral	Alkaline	Positive	Negative
Horse...............	19*	0	0	19
Pig..................	31	0	0	31
Cow.................	20	0	0	20
Sheep...............	22	0	0	22
Raw sewage..........	14	5	5	14
Septic sewage.........	16	4†	4†	16
Man.................	25	0	0	25

* The figures indicate the number of organisms.
† One organism did not give a Voges-Proskauer reaction, and was not alkaline to methyl red until the 7th day.

tures from raw and septic sewage 9, or about 23%, gave these two reactions, while not a single culture out of the 117 from the fecal sources was positive in the Voges-Proskauer test, or alkaline to methyl red.

What is the origin of these Voges-Proskauer-positive, methyl-red-negative organisms found in sewage, which are presumably very rarely found in the feces of cows, pigs, horses, sheep, and man? The scarcity of Voges-Proskauer-positive colon bacilli in feces confirms previous work by MacConkey, who, in the examination of 205 colon-like organisms isolated from 22 samples of human stools, obtained positive tests in only 4 cultures, 3 of which were from a single sample. Clark and Lubs have shown that the colon-like organisms occurring on grains were characterized by an alkaline reaction to methyl red. The work here presented agrees with their findings to the extent that methyl-red-negative organisms are apparently of non-fecal origin.

Whether B. coli will multiply on grain or not, it is evident that the organisms must first reach the grain from some extraneous source. This source is in all probability the soil. Investigations are now under way at this laboratory to determine whether the incidence of Voges-Proskauer-positive colon bacilli is considerably higher in soil than elsewhere.

A study of 13 organisms in this collection which form acetyl-methylcarbinol from dextrose as to their ability to produce gas in carbohydrates, gave the results shown in Table 5. Dextrose, galactose, maltose, lactose, raffinose, and saccharose were fermented by all with the production of gas and acid. Inulin was not attacked by any. Glycerin was

TABLE 5

Fermentation of Carbohydrates by Voges-Proskauer-positive, Methyl-red-negative Organisms

Culture	Source	MacConkey Type	Saccharose	Raffinose	Dulcite	Glycerin	Salicin	Inulin
96	Raw sewage.........	Communior....	+	+	+	+	+	—
99	Raw sewage.........	Aerogenes......	+	+	—	—	—	—
102	Raw sewage.........	Aerogenes......	+	+	—	+	+	—
107	Raw sewage.........	Communior....	+	+	+	+	+	—
108	Raw sewage......	Aerogenes......	+	+	—	+	+	—
112	Septic sewage........	Aerogenes......	+	+	—	+	+	—
117	Septic sewage........	Aerogenes......	+	+	—	+	+	—
119	Septic sewage........	Communior....	+	+	+	+	+	—
128	Septic sewage........	Aerogenes......	+	+	—	+	+	—
160	Amer. Museum of Natural History	Communior....	+	+	+	+	+	—
166	Amer. Museum of Natural History	Aerogenes*.....	+	+	+	+	+	—
167	Amer. Museum of Natural History	Cloacae........	+	+	—	—	+	—
168	Amer. Museum of Natural History	Mucosus capsulatus	+	+	—	+	+	—

* Reacts in dulcite like B. coli [communior] but was labeled B. [lactis] aerogenes.

fermented by 11, or 84.6%. Twelve organisms, or 92.3%, produced gas in salicin, while only 38.5% fermented dulcite. Kligler[10] points out that fermentation of salicin is more closely correlated with the Voges-Proskauer reaction than is gas-production in dulcite. I find, as he did, that organisms which give the Voges-Proskauer reactions almost always break down salicin with the liberation of gas, while no correlation exists with dulcite. In this collection of 167 organisms, there are a large number which ferment salicin and do not give the Voges-Proskauer reaction.

Durham[11] observed that the Voges-Proskauer reaction was characteristic of B. [lactis] aerogenes. He noted that this reaction was given by all cultures

[10] Jour. Infect. Dis., 1914, 15, p. 187.
[11] Jour. Exper. Med., 1900-1901, 5, p. 353.

which he regarded as belonging to the B. [lactis] aerogenes group, but not by the organisms in other groups. MacConkey,[7] after recording the opinions of Theobald Smith, Strong, Durham, Muir, and Ritchie, Jordan, Horrocks, Paul Clairmont, Lehman and Newman, and others, concluded that "taking all these opinions together, the B. lactis aerogenes (Escherich) might be described as a non-motile, gram-negative, non-liquefying, bacillus; a facultative anaerobe, producing acid and clotting in milk, fermenting glucose, lactose, cane sugar, and maybe also starch and inulin, and giving the Voges-Proskauer reaction." In short, he characterized B. [lactis] aerogenes as a colon-like organism which fermented saccharose and formed acetyl-methyl-carbinol from dextrose, but, in 1909 he re-defined this organism specifying that dulcite was not fermented.

The work of Rogers and his colleagues in the U. S. Department of Agriculture,[3] as well as that of Howe,[9] Kligler,[10] and myself, indicates that dulcite-fermentation (except possibly in the B. coli [communis] group) is not well correlated with other characters. I should therefore disregard the dulcite-fermentation and, returning to MacConkey's first characterization of B. [lactis] aerogenes, re-define this organism as a non-sporeforming, non-liquefying, gram-negative short rod, which forms acid and gas from dextrose, lactose, saccharose, and probably raffinose, and acid and clot in milk. Salicin and glycerin are almost always fermented but dulcite only occasionally. Its reactions in appropriate dextrose media, where it forms acetyl-methyl-carbinol (Voges-Proskauer-positive), gives an alkaline reaction with methyl red, and breaks down the sugar with liberation of carbon dioxid in great excess over hydrogen, are particularly distinctive.

It is apparently this type of organism that the investigations of Rogers and his associates[3] showed to be so characteristic of grain. The sanitary significance of the Voges-Proskauer reaction is therefore very evident. Colon-like organisms which form acetyl-methyl-carbinol from dextrose are characteristically of non-fecal origin.

Rogers and his co-workers emphasized the $CO_2 : H$ ratio but did not employ the Voges-Proskauer test. MacConkey,[7] Bergey and Deehan,[12] Kligler,[10] and many others who record the Voges-Proskauer reaction, did not determine the gas ratio. Clark and Lubs[5] showed that the gas ratio is correlated with acidity and alkalinity to methyl red, and this paper indicates that the methyl-red reaction is in turn correlated with the Voges-Proskauer reaction.

Altho in this collection only those cultures which were alkaline to methyl red gave the Voges-Proskauer reaction, this absolute correlation should not be overemphasized, as the number of organisms was small.

[12] Jour. Med. Research, 1908, 19, p. 175.

Other collections should be studied to determine the correlation between these two tests.

SUMMARY AND CONCLUSIONS

A study of 167 colon-like organisms isolated from various sources including the horse, cow, pig, sheep, man, raw sewage, septic sewage, and standard cultures showed that:

There is a better correlation between saccharose-fermentation and the source than between the source and saccharose-dulcite-fermentation.

For the methyl-red reaction with an incubation period of from 3 to 5 days, there is little choice between 30 and 37 C.; but if incubation is reduced to 2 days, the body temperature is preferable.

The methyl-red and Voges-Proskauer reactions are correlated. The organisms which were alkaline to methyl red gave a positive Voges-Proskauer test and vice versa, and in many other characters they resembled B. [lactis] aerogenes.

The cultures which formed acetyl-methyl-carbinol from dextrose almost always fermented salicin and glycerin, but attacked dulcite only occasionally.

Organisms that give the Vosges-Proskauer reaction are rarely found in feces.

The production of acetyl-methyl-carbinol from dextrose serves as an available basis for the comparison of recent work on the colon-aerogenes group of bacteria with the numerous results of previous investigations.

For the Voges-Proskauer reaction, the dextrose peptone di-potassium phosphate medium of Clark and Lubs[5] gives a more distinct test than that given by dextrose broth.

The Voges-Proskauer reaction, like the high gas ratio and the alkalinity to methyl red, is characteristic of non-fecal strains, and it may therefore be of considerable sanitary significance.

THE BACTERICIDAL AND FUNGICIDAL ACTION OF COPPER SALTS *

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS, XV

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From the middle of the nineteenth century to the present day the value of copper salts as germicidal or as therapeutic agents has been a subject of interest.

Historical Review

In 1853 the French government recorded its belief in the harmfulness of copper as a dietary constituent[1] by the passage of a law (afterward repealed) prohibiting its addition to canned vegetables. Apparently the copper served for the maintenance of the green color of the vegetables, tho Johnson and Copeland[2] suggest that it may have served a double purpose: "Copper sulphate is a powerful germicide. It has for years been added to canned meats and vegetables to prevent fermentation by bacteria and other organisms."

For at least the last quarter of the nineteenth century, the belief in the germicidal action of copper was particularly flourishing. The early observations of its effect were made almost simultaneously on algae, fungi, and bacteria. During the years 1880 to 1883 Burq[3] reported the results of an investigation, begun 30 years earlier, comprising a mass of data acquired by personal visits to all sorts of establishments engaged in the manufacture of copper and other metals, by extensive communication with the officers of workingmen's unions, working men themselves, proprietors of foundries, physicians, and mayors of towns where metal-working was the principal industry, by correspondence with English, Swedish, and Russian ambassadors, with the directors of Siberian mines, and with all possible individuals engaged in, or connected with, the mining and manufacture of copper—all of which led him to conclude that impregnation of the workmen with copper conferred on them a singular immunity to cholera. He found them equally immune to typhoid infection, and suggested that a similar immunity might exist toward diphtheria and smallpox. (These findings have been largely discredited as a result of more recent investigations.) In 1883 Walker[4] published a monograph, written 20 years earlier, in which the verdict runs: "At all events the immunity of this class

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[1] Kraemer: Am. Jour. Pharm., 1905, 77, p. 265.

[2] Jour. Infect. Dis., 1905, Suppl. 1, p. 327.

[3] Bull. de l'Acad. de méd., 1880, 9, p. 239. Compt. rend. Acad. d. sc., 1882, 95, p. 862. Compt. rend. Soc. de biol., 1883, 5, p. 532.

[4] The Prophylactic Power of Copper in Epidemic Cholera, 1883. Quoted by Moore, Bull. U. S. Dept. of Agriculture, Bur. Plant Ind., 76, p. 52.

of men [copper-workers] for cholera is a remarkable and positive fact. I have for a long time made inquiries in this matter, and cannot as yet learn that a single case has occurred among them." In 1876, according to Green,[5] von Bucholtz established the fact of the inhibitory effect of copper sulfate, finding that 0.75% checked all bacterial development in nutrient media. Green[5] himself investigated the disinfectant action of certain soluble copper compounds— the chlorid, nitrate, acetate, aluminate, phenolsulfonate, and sulfate (C. P., crude, and ammoniacal)—on various substances and organisms, and found them efficient, for the most part, in the order given. One hour's exposure to 5% copper chlorid killed cholera and typhoid organisms, while 3 hours' exposure was necessary in the case of Staphylococcus aureus. Spore-free anthrax bacilli were killed in 5 minutes by 5% copper chlorid, and anthrax spores were retarded in their development by copper salts, and killed by exposure to 5% copper chlorid for 27 days.

As a fungicide, copper owes its chief prominence to Millardet's use of copper sulfate and lime in the Bordeaux mixture as protection against Plasmopora, which wrought such havoc in the French vineyards in 1883. At about the same time Naegeli[6] observed what he termed the "oligodynamic" effect of copper, one part of which in one billion parts of water was extremely toxic to Spirogyra.

From this time on, experimental investigation of the efficiency of copper in various forms seems to have thriven. Behring[7] found copper sulfate a "very good disinfecting agent." Israel and Klingmann[8] continued the study of the effect of copper on Spirogyra, and on bacteria, using B. typhosus, B. coli, and Vibrio cholerae. In their experiments the bactericidal effect was obtained by exposing the organisms for different lengths of time to water in which small pieces of copper foil had been left for 24 hours. All manifested delay of growth after a half hour's exposure, while 2 hours' exposure killed B. typhosus, and 3 hours' exposure, B. coli and V. cholerae. Increase of temperature doubled the toxic effect, halving the time required. Bolton[9] found that when metals, and copper among them, were laid on agar plates inoculated with organisms, zones of inhibition were produced. He also discovered that in sufficient dilution copper exerted a stimulative effect. Krönig and Paul[10] in their work on disinfection found that copper chlorid, even in so concentrated a solution as 13%, failed to kill anthrax spores during a 10 days' exposure, tho 3.4% auric chlorid killed them in 33 hours, and 1.69% mercuric chlorid in 14 minutes.

In 1904 the practical use of copper as a disinfectant received a fresh impetus from the publication of investigations which had been prosecuted for 2 years by Moore and his co-workers in the U. S. Department of Agriculture.[11] These investigations were directed primarily toward the destruction of those algal growths which produce the various disagreeable tastes and odors so common in many water supplies during the summer months. Moore found small amounts of copper sulfate very efficient in ridding water supplies of algae, while pathogenic bacteria (notably typhoid) also disappeared in many instances. Moore's work aroused widespread experimentation, with varying results. Pen-

[5] Ztschr. f. Hyg. u. Infektionskrankh. 1895, 13, p. 495.
[6] Neue Denkschriften der Schweiz. Naturforsch. Gesellsch., 1893-98, 33-34, 2nd paper.
[7] Ztschr. f. Hyg. u. Infektionskrankh., 1890, 9, p. 395.
[8] Virchow's Arch. f. path. Anat., 1897, 147, p. 293.
[9] Internat. Med. Mag., 1894-5, 3, p. 812.
[10] Ztschr. f. Hyg. u. Infektionskrankh., 1897, 25, p. 1.
[11] Bull. U. S. Dept. of Agriculture, Bur. Plant Ind., 64, p. 76.

nington[12] and her assistants found that in very clean brightly polished copper vessels typhoid bacilli were soon killed, sometimes in less than 2 hours, while Kraemer[1], after 2 years of experimentation declared: "I have found that when copper foil is allowed to remain in distilled water from one to five minutes sufficient copper is dissolved by the water to kill typhoid organisms within two hours."

Clark and Gage,[13] on the other hand, decided that copper sulfate was of little practical importance, since sterilization required amounts so large as to render the water unpalatable. Johnson and Copeland[2] found reduction of organisms in sewage, both as it was when collected and as it was with the addition of typhoid bacilli. Feldt[14] found that in asparagin media both copper sulfate and copper chlorid inhibited the growth of tubercle bacilli only in concentrations of from 1 : 5000 to 1 : 50,000. Von Linden,[15] however, stated that when added to artificial protein-containing media, copper salts exercised an inhibitory effect on vigorous cultures of tubercle bacilli even in dilution of 1 : 1,000,000. At 1 : 100,000, all, even large clumps, were killed, so that transfers failed to grow. DeWitt and Sherman,[16] on the other hand, found vigorous growth developing on tubes containing 1 part of copper to 1,000,000 parts of media, while inhibition occurred at a concentration of 1 : 100,000 and killing only after 24 hours' exposure to 5% copper.

In addition to its use for the purification of drinking water, some attempt has been made at sewage-treatment with copper sulfate, but the excessive amount of organic matter in sewage causes precipitation of the copper, thereby diminishing its efficiency. It has also been employed for the removal of bacteria in swimming pools, more or less successfully.

Since its introduction by Millardet, copper in some form continues a common ingredient of sprays and fungicides. It is frequently employed in sterilization of seeds against adhering or infecting fungi. In this connection it is of interest that one strain of Penicillium, a fungus often found contaminating cultures of germinating seeds, is particularly resistant to the action of copper. Pulst[17] found that P. glaucum withstood 10% copper sulfate, while Hollrung[18] stated that Trabut had described a P. cupricum, (apparently a mutant of P. glaucum) which grew in concentrated copper sulfate solution. It may be mentioned in passing that the stock solution of copper amino-acids in this laboratory, containing 1.8% copper, was regularly overgrown with Penicillium.

The idea of employing copper as a therapeutic agent has long existed. Burq[3] and Walker[4] recommended the use of copper in the therapy of typhoid. Green[5] advised the treatment of wounds with copper chlorid. Wilcke[19] used copper acetate internally to free typhoid-carriers of the bacilli, and with some success. Springer and Springer[20] found that copper salts delayed or inhibited the action of putrefactive organisms, and so might have a therapeutic value. Loeb[21] and

[12] Science, 1905, 21, p. 611.

[13] Jour. Infect. Dis., 1906, Suppl. 2, p. 175.

[14] Deutsch. med. Wchnschr., 1913, 39, p. 549.

[15] Münch. med. Wchnschr., 1914, 61, p. 586.

[16] Jour. Infect. Dis., 1914, 15, p. 245.

[17] Quoted without reference by Pfeffer, Physiology of Plants, Eng. ed., 1903, 2, pp. 260, 262, 266.

[18] Handbuch d. chemische Mittel gegen Pflanzenkrankheiten, 1898, p. 75.

[19] Ztschr. f. Medizinalbeamte., 1913, 26, p. 772.

[20] Jour. Ind. and Eng. Chem., 1909, 1, p. 676.

[21] Jour. Am. Med. Assn., 1913, 60, p. 1857. Jour. Exper. Med., 1914, 20, pp. 169, 180, 503, 522. Arch. f. Int. Med., 1915, 15, p. 974.

his co-workers are experimenting with injections of colloidal copper in the treatment of cancer, while von Linden and her co-workers have reported favorably on the action of copper in the treatment of tuberculosis. Luton[22] insists on the therapeutic value of copper compounds in the form of pills, salves, or injections, in all kinds of tuberculous infections, however severe. According to his statement the use of copper for the treatment of tuberculosis dates back to 1885, and has continued spasmodically ever since, frequently effecting a cure in the early stages and exercising a palliative effect under all circumstances. Corper,[23] however, found that copper salts injected into tuberculous guinea-pigs or rabbits had no effect on the course of the disease, a statement confirmed by Moewes and Jauer,[24] by Kaiser,[25] and others.

A large part of the prejudice against the use of copper, especially for the purification of drinking water, is due, apart from any unpleasant flavor which may result, to a general notion that copper is poisonous to animals. Investigation has proved that this metal is constantly present in the animal body, and occurs naturally in a large number of those foods which combine to form the usual diet of mankind. That its presence in the diet is practically harmless has been held by many physiologists. Galippe[26] and Kraemer,[1] by using copper utensils for the preparation and serving of food, for periods of many months, demonstrated the innocuousness of small amounts of copper in the diet. Many pharmacologists regard it as innocuous, and Holland, in correspondence with Kraemer, stated that to produce poisoning the administration of copper must be deliberate and in large amounts, and that the physical action would cease with cessation of the application of the salt.

The present work, which was undertaken because of the conflicting testimony of investigators as to the bactericidal power of copper, falls naturally into two divisions: first, investigation of the killing power of the sulfate and the chlorid of copper, and second, a study of their inhibitory action. The organisms used have been chiefly bacteria— B. coli, B. typhosus, B. prodigiosus, B. tuberculosis, and Staphylococcus aureus; but for comparison the behavior of certain fungi (a baker's white yeast, a pink torula, Aspergillus niger, and Penicillium decumbens), was also observed.

TECHNIC

In the earlier experiments Krönig and Paul's garnet method was followed: A suspension of organisms was allowed to dry on glass beads, which were then exposed to the action of various dilutions of copper. After washing with ammonium sulfite to neutralize any adherent copper solution, and then with water, a definite number of garnets were placed in test tubes, thoroughly shaken in a shaking machine, and then plated with 10 c.c. of agar. More consistent results were obtained with a modification of the method devised by Anderson and McClintock, which was accordingly substituted for the garnet method in the later experiments. The procedure was as follows: The growth

[22] La Province med., 1912, 23, p. 549.
[23] Jour. Infect. Dis., 1914, 15, p. 518.
[24] München. med. Wchnschr., 1914, 61, p. 1439.
[25] Therap. Monatsh., 1914, 28, p. 748.
[26] Ann. d'hyg. pub., 1878, 50, p. 426.

from a 48-hour agar-slant culture of the organism was mixed with sterile normal salt solution, or with sterile water distilled in glass, to give a faintly opaque suspension. Of this suspension 0.1 c.c. was added to 15 c.c. of each of the copper dilutions and left for various lengths of time, exposures of $2\frac{1}{2}$, and 15 minutes being finally selected for the experimental routine, tho in certain experiments the time was extended to 2, 4, and even 24 hours. One drop of the inoculated copper solution was then placed in a tube containing 10 c.c. of sterile distilled water and 1 c.c. of this dilution was used with 10 c.c.

TABLE 1

AVERAGE RESULTS OF 15-MINUTE EXPOSURES OF VARIOUS ORGANISMS TO THE ACTION OF COPPER

Organism	CuCl₂					
	5%	1%	0.1%	0.01%	0.001%	0.0001%
Staphylococcus aureus.....	0	705	4,752	5,792	11,824	16,899
Bacillus coli................	2	3	16	868	294	16,962
Bacillus prodigiosus.......	0	0	1	0	1	1,930
Yeast (white)..............	0	63	67	318	2,214	5,874
Torula (pink)..............	0	94	129	703	1,036	7,168
Aspergillus.................	278	798	948	1,043	1,132	......
Penicillium decumbens.....	615	1,211	......	1,771	......	2,112

TABLE 2

THE EXTENT OF VARIATION IN THE ACTION OF COPPER ON STAPHYLOCOCCUS AUREUS AND ON BACILLUS COLI IN AN EXPOSURE OF 15 MINUTES' DURATION

Experiment		CuCl₂					
		5%	1%	0.1%	0.01%	0.001%	0.0001%
S. aureus.....	1............	......	......	......	......	......	......
	2............	......	......	......	......	......	......
	3............	......	......	......	......	......	......
	4............	......	......	......	......	......	......
	5............	......	......	......	......	......	......
	6............	......	......	......	......	......	......
	7............	0	0	0	36	6,391	1,496
	8............	0	2,116	14,256	17,339	23,142	38,184
	9............	0	0	0	1	5,940	11,016
B. coli........	1............	......	......	......	......	......	......
	2............	......	......	......	......	......	......
	3............	......	......	......	......	......	......
	4............	......	......	......	......	......	......
	5............	0	0	14	21	911	42,370
	6............	1	1	28	3,210	77	24,124
	7............	0	1	12	239	178	921
	8............	6	10	9	3	10	433

of agar, for each of 4 plates. The further dilution of the inoculated copper solution by transfer to 10 c.c. of water instead of directly to the plate, was designed to avoid any inhibitory effect which might result from the presence of so large an amount of copper in the agar, since in these experiments it was the killing action of copper which was of primary interest. Controls were treated in the same way throughout, sterile distilled water or sterile normal salt solution being substituted for the copper dilution.

For convenience in the comparison of results, the amounts of either salt used in making the dilutions were so calculated as to give corresponding per-

centages of copper. Dilutions containing 5, 1, 0.1, 0.01, 0.001, 0.0001% of copper were used.

All plates were counted after 48 hours in the incubator or at room temperature, according to the organism.

In Table 1 are given the average results of the various experiments with the different organisms (by the Anderson and McClintock method, modified). It will be observed that the organisms manifested a certain

TABLE 1—*Continued*

AVERAGE RESULTS OF 15-MINUTE EXPOSURES OF VARIOUS ORGANISMS TO THE ACTION OF COPPER

CuSO₄							Control 0.9% NaCl
5%	1%	0.1%	0.01%	0.001%	0.0001%	0.00001%	
86	389	4,838	2,415	9,730	17,554	4,326	12,208
2	70	211	1,541	3,826	26,379	33,208	20,589
0	0	1	2	38	24,116	84,213	45,072
0	5	132	135	777	4,341	6,116	4,589
0	645	831	1,537	1,483	2,782	4,061	5,988
405	495	286	386	1,134	1,071	....	689
646	1,096	......	1,284	......	1,855	....	1,949

TABLE 2—*Continued*

THE EXTENT OF VARIATION IN THE ACTION OF COPPER ON STAPHYLOCOCCUS AUREUS AND ON BACILLUS COLI IN AN EXPOSURE OF 15 MINUTES' DURATION

CuSO₄							Control
5%	1%	0.1%	0.01%	0.001%	0.0001%	0.00001%	
103	240	228	337	490	6,967	580	874
214	168	16,518	......	......	......	....	10,928
29	63	57	......	......	......	....	578
0	0	2,549	6,312	18,969	28,141	8,072	10,076
2	960	5,143	11,584	14,236	22,448	12,936	5,790
2,137	3,175	22,176	42,078	31,860	57,548	37,175	8,946
......	......	......	......	......	......	....	12,000
......	......	......	......	......	......	....	46,528
......	......	......	......	......	......	....	18,834
0	0	3	26	204	479	744	1,091
0	1	5	90	1,284	37,591	39,312	39,924
0	45	1	5,994	11,414	55,004	58,000	39,857
6	235	33	56	2,403	12,345	42,776	28,392
......	......	......	......	......	......	....	30,919
......	......	......	......	......	......	....	23,014
......	......	......	......	......	......	....	1,030
......	......	......	......	......	......	....	490

specificity in their reaction to copper, some being more susceptible than others. B. prodigiosus, for instance, appears little resistant to the action of copper, bacilli being practically all killed by dilutions of copper, as the sulfate, up to 1 : 10,000, and as the chlorid, up to 1 : 100,000; while Staphylococcus aureus is relatively resistant, 1% copper as chlorid and 5% as sulfate failing to kill every organism. With the exception of Staphylococcus, Aspergillus, and Penicillium,

5% copper in either form killed all the organisms studied. A slight difference in efficiency in favor of the chlorid is noticeable between the two salts.

That Staphylococcus aureus is a relatively resistant organism was noted by Green, who found that it succumbed to 2.5% copper chlorid (0.9% copper) only after 5 hours' exposure and to the same percentage of copper sulfate (0.6% copper) after 1 day. Bolton observed the same resistance; for 50 minutes' contact of metallic copper and an inoculated agar plate was required in the case of Staphylococcus aureus to produce a clear zone, while from 1 to 5 minutes' exposure was sufficient in the case of B. coli, B. typhosus, and V. cholerae. Furthermore, according to Bolton, the resistance of the same organism varied at

TABLE 3

RESULTS OF THE INHIBITORY ACTION OF COPPER ON VARIOUS MICRO-ORGANISMS

Organism	CuCl₂			
	0.1%	0.01%	0.001%	0.0001%
Staphylococcus aureus......	0	0	3,534	8,239
Staphylococcus citreus......	0	0	21,616	24,916
Bacillus coli................	0	16,613	47,839	39,931
Bacillus prodigiosus.........	0	12,634	15,023	15,915
Yeast (white)...............	0	9,731	11,267	11,725
Torula (pink)..............	0	11,299	25,900	18,420
Aspergillus.................	0	204	167	157
Penicillium...:.............	0	1,142	1,762	1,502
B. tuberculosis..............	0	0	0	+++

different times—a phenomenon met with in the experiments here reported. Table 2 illustrates the extent of variation in 9 experiments with Staphylococcus aureus and 8 with B. coli. While there is a general similarity of behavior from experiment to experiment, there are irregularities of progression and variations in the results obtained under apparently identical conditions (even 5% copper failing to kill Staphylococcus aureus in one instance)—variations which are largely obliterated on averaging the results.

For the study of the inhibitory action of copper such amounts of stock solutions of the chlorid and the sulfate were taken as, added to the 10 c.c. of agar used for plating, would give the desired dilution. (These amounts were introduced into the plate rather than added to the tube of agar, since precipitation of copper occurred on contact of the copper solution and the agar and only by rapid cooling after mixing could this precipitation be prevented or reduced to a minimum.) One-tenth cubic centimeter of the suspension of the organism was then

added. To avoid any preliminary inhibitory action of copper on the organism before the introduction of the agar not only were the copper dilution and the bacterial suspension introduced as far apart as the limit of the plate would permit, but the dilution was so calculated as to make the required amounts of stock solution as minute as was consistent with accuracy of measurement (0.1 or 0.2 c.c.). In the experiments for inhibitory action the dilutions contained 0.1, 0.01, 0.001, and 0.0001% copper.

Table 3 contains the average results of these experiments. It is obvious that, in general, inhibition occurs with a lower concentration of copper than that at which killing occurs. B. tuberculosis, which was included in this table, is more susceptible than the other organisms

TABLE   3—*Continued*

RESULTS OF THE INHIBITORY ACTION OF COPPER ON VARIOUS MICRO-ORGANISMS

CuSO₄				Control
0.1%	0.01%	0.001%	0.0001%	
0	0	0	47,178	17,870
0	0	39,288	29,792	33,509
0	0	45,305	59,961	46,252
0	0	71,718	69,708	34,914
0	0	13,800	12,372	19,072
0	1,628	30,816	20,484	18,948
0	150	212	95	180
0	1,707	1,758	2,222	1,264
0	0	0	+++	+++

used, its growth being inhibited by dilutions of 1 part copper, in either form, to 100,000 of media. Next in order, Staphylococcus aureus is inhibited by copper as chlorid in dilution of 1 : 10,000 and by copper as sulfate in dilution of 1 : 100,000. With the exception of Penicillium, against which the chlorid is less efficient than the sulfate, and of Aspergillus, on which the two salts have an equal effect, copper in the form of the sulfate has a stronger inhibitory action than in the form of the chlorid. Dilutions of 1 : 1000 inhibit growth of all the organisms. Dilutions of 1 : 10,000 inhibit Staphylococcus aureus, B. coli, B. prodigiosus, and the white yeast. In the case of the pink yeast and the two moulds the growth is diminished somewhat, but inhibited only by the stronger concentration.

It is noticeable that there is an exception to the rule that inhibition is accomplished by a lower concentration than is required to kill. B. prodigiosus is inhibited by dilutions of copper of 1 : 1000 as chlorid, and of 1 : 10,000 as sulfate, while dilutions of copper of 1 : 100,000 as

chlorid, and of 1 : 10,000 as sulfate, kill in 15 minutes. Since, however, repeated experiments give the same result, the peculiarity appears to be intrinsic in the organism. B. coli also forms a partial exception to the rule.

Inasmuch as the results thus far given were less favorable as regards the disinfectant value of copper than were the results in Moore's work,[11] other experiments were undertaken to see whether even under laboratory conditions, with longer exposures, comparable results might be obtained. Accordingly, 200 c.c. of ordinary tap water which had been allowed to flow for one-half hour were placed in each of several sterile

TABLE 4

Results of the Action of Copper on the Bacterial Content of Tap Water

Time of Exposure		$CuCl_2$				
		0.1%	0.01%	0.001%	0.0001%	0.00001%
In sterile flasks...	1 hr.	0	3	4	45	1,710
	3 hr.	0	3	9	3,759	31,287
	24 hr.	0	12	4	333	7,232
	48 hr.	0	1	4	3,924	7,429
	96 hr.	0	1	14	25,459	92,926
	1 wk.	0	2	21,325	100,753	69,691
	2 wk.	0	10	1,267	7,990	125,390
	3 wk.	0	2	7,082	2,873	37,569
	4 wk.	0	10	1,243	33,828	127,311
	5 wk.	2	4	1,009	25,208	243,915
	6 wk.	0	3	37,041	29,989	857,600
In large open crocks..	1 hr.	0	14	371	13,819	14,553
	3 hr.	1	32	714	5,306	11,760
	24 hr.	1	88	237	4,143	19,729
	48 hr.	1	61	106	10,035	15,797
	96 hr.	0	8	3,894	19,699	31,060
	1 wk.	3	15	167,039	25,049	104,803
	2 wk.	0	151	169,958	62,847	227,563

flasks, and sufficient amounts of stock solutions of copper chlorid and of copper sulfate added to make dilutions containing 0.1, 0.01, 0.001, 0.0001, and 0.00001% copper. The number of organisms present was obtained by making a control of the water alone. Plates were made after 1, 3, 24, and 96 hours, and after 1, 2, 3, 4, 5, and 6 weeks. Similarly, to approximate even more closely the conditions in an open reservoir, large earthen crocks, holding approximately 4 liters, were filled with tap water and the requisite amounts of chlorid or of sulfate added to make the dilutions desired. The crocks were left uncovered for 2 weeks in a sunny laboratory. The results are embodied in Table 4.

While 1 part copper to 1000 of water kills all the ordinary water organisms, and 1 part copper to 10,000 of water causes a great reduction in their number, they are little affected by higher dilutions of cop-

per, increasing in number rather than decreasing on long exposure. The ordinary water organisms, however, are relatively innocuous; it is only the pathogenic bacteria the presence of which in water is of special sanitary significance.   Is copper as toxic to these as Moore[11] and his contemporary workers claimed?   To test this point 0.5 c.c. of a suspension of B. coli was added to flasks containing sterile tap water and the same amounts of copper as before, and plates were made at intervals, up to 3 weeks.   From Table 5 it is seen that copper as sulfate in dilutions of 1 : 1,000,000 killed all organisms in 24 hours, and in dilutions of 1 : 10,000,000, as both sulfate and chlorid, in 96 hours.

TABLE   4—*Continued*

RESULTS OF THE ACTION OF COPPER ON THE BACTERIAL CONTENT OF TAP WATER

$CuSO_4$					Control
0.1%	0.01%	0.001%	0.0001%	0.00001%	
0	2	4	41	6,505	8,835
0	1	4	3,533	41,104	17,037
0	8	15	173	9,223	78,983
0	2	2	2,079	12,708	23,690
0	1	198	40,427	68,051	15,138
0	3	34,504	62,782	39,278	21,103
0	15	6,554	192,288	75,993	38,458
0	1	19,863	106,062	67,128	15,299
0	7	21,293	181,261	91,651	29,961
2	35	24,116	124,933	137,697	56,522
0	1	1,642	124,336	617,876	114,746
0	11	1,325	6,517	28,195	14,374
1	13	63	3,477	9,221	16,732
1	15	13	8,174	20,025	7,984
1	11	38	7,915	16,140	3,589
0	8	305	14,607	18,290	8,518
2	31	23,301	36,864	105,795	5,197
0	47	129,601	55,460	279,288	6,888

In making the experiments it was observed that a heavy precipitation occurred on the addition of the copper to the tap water, making it impossible to tell the actual amount of copper in a solution producing a given effect.   To overcome this difficulty, distilled water was used instead of tap water, but since controls in distilled water died, the presence of traces of copper was suspected.   Analyses of the water verified this suspicion.   That ordinary distilled water contained traces of metals, particularly of copper, had been noticed by Ringer, who ascribed the toxic action of distilled water on living organisms to this constituent. For this reason, in subsequent experiments water redistilled in glass was employed.   In twice-distilled water the controls lived indefinitely, plates made 8 weeks after inoculation showing but slight reduction. Colon bacilli exposed to various amounts of copper failed to survive for 24 hours in dilutions of 1 : 1,000,000.

The length of time required for complete sterilization of twice-distilled water in dilutions of 1 : 1,000,000, as compared with that required for tap water in the same dilution, suggests the possible presence of copper ordinarily in the latter. Analyses made in the chemical laboratory of the University of Chicago showed this to be a fact.

TABLE 5

Results of the Action of Copper on B. Coli in Sterile Tap Water, and in Twice-distilled Water

Time of Exposure		CuCl₂				
		0.1%	0.01%	0.001%	0.0001%	0.00001%
In sterile tap water	1 hr.	0	1,403	229,517	613,651	781,151
	3 hr.	0	4	88,602	96,765	566,282
	24 hr.	0	0	316	440	69,616
	48 hr.	0	0	0	0	5,682
	96 hr.	0	0	0	0	0
	1 wk.	0	0	0	0	0
	2 wk.	0	0	0	0	0
	3 wk.	0	0	0	0	0
In twice distilled water	30 min.	236	42,437	60,648	315,309	981,441
	1 hr.	9	2,881	36,707	668,427	695,331
	3 hr.	0	69	23	9,567	757,266
	24 hr.	0	0	0	0	22,536
	48 hr.	0	0	0	0	4,472
	1 wk.	0	0	0	0	87
	2 wk.	0	0	0	0	1
	3 wk.	0	0	0	0	0
	4 wk	......	......	....	....	....
	5 wk.	......	......	....	....	....
	6 wk.	......	......	....	....	....
	7 wk.	......	......	....	....	....
	8 wk.	......	......	....	....	....

TABLE 6

Results of the Action of Copper on Natural B. Coli in Twice-distilled Water (glass), and in Sterile Tap Water

Time of Exposure		CuCl₂				
		0.1%	0.01%	0.001%	0.0001%	0.00001%
In twice distilled water	1 hr	0	0	2	94,793	1,224,496
	3 hr.	0	0	0	32,714	579,080
	24 hr.	0	0	0	1	355,827
	48 hr.	0	0	0	0	291,936
	96 hr.	0	0	0	0	242,402
	1 wk.	0	0	0	0	200,896
	2 wk.	0	0	0	0	186,192
In sterile tap water	1 hr.	0	35	151,024	599,401	831,046
	3 hr.	0	2	77,763	100,606	665,801
	24 hr.	0	1	8	36	95,111
	48 hr.	0	0.6	0	26	184,563
	96 hr.	0	0	0	26	198,146
	1 wk.	0	0	0	4	55,946
	2 wk.	0	0	0	0	8,914

Since Clark and Gage[13] had found that B. coli living in water resisted the action of copper sulfate in dilution of 1 : 10,000 for 103 days, while laboratory cultures were killed in 24 hours, our experiment

was repeated with a strain of B. coli recently isolated from drinking water. Their statement appears justifiable, since (Table 6) this water strain of Bacillus coli survived for 1 week in dilutions of 1 : 1,000,000, and when the experiment was brought to an end, after 2 weeks, the organisms were still numerous, tho their number was much reduced,

TABLE   5—*Continued*

RESULTS OF THE ACTION OF COPPER ON B. COLI IN STERILE TAP WATER, AND IN TWICE-DISTILLED WATER

CuSO₄					Control
0.1%	0.01%	0.001%	0.0001%	0.00001%	
0	5,534	259,765	560,258	740,182	1,155,975
0	2	22,133	17,121	297,777	1,118,864
0	0	0	0	34,720	1,191,685
0	0	0	0	5,299	672,956
0	0	0	0	0	1,783,621
0	0	0	0	0	588,351
0	0	0	0	0	530,643
0	0	0	0	0	443,808
30,101	6,841	15,992	88,104	603,104	1,389,925
219	1,011	1,760	32,766	843,831	1,395,305
0	9	21	2,158	324,191	1,204,917
0	0	0	0	7,480	1,263,993
0	0	0	0	1,024	1,159,845
0	0	0	0	16	1,027,096
0	0	0	0	0	857,784
0	0	0	0	0	968,931
....	....	....	....	....	1,157,815
....	....	....	....	....	1,222,720
....	....	....	....	....	1,310,481
....	....	....	....	....	739,159
....	....	....	....	....	208,765

TABLE   6—*Continued*

RESULTS OF THE ACTION OF COPPER ON NATURAL B. COLI IN TWICE-DISTILLED WATER (GLASS), AND IN STERILE TAP WATER

CuSO₄					Control
0.1%	0.01%	0.001%	0.0001%	0.00001%	
0	0	1	104,286	1,455,248	3,971,680
0	0	1	39,588	784,973	3,785,953
0	0	2	2	381,606	4,194,546
0	0	0	0	401,923	4,680,873
0	0	0	0	464,165	4,192,560
0	0	0	0	374,110	1,655,240
0	0	0	0	99,788	1,478,666
0	40	233,008	530,951	867,479	1,187,658
0	12	9,652	105,388	680,907	1,389,104
0	0	1	11	82,106	1,411,281
0	0	2	3	184,343	1,435,859
0	0	0.6	6	110,294	1,418,373
0	0	0.5	1	37,923	1,473,970
0	0	0	0	25,054	1,575,318

in a dilution of 1 : 10,000,000. In twice-distilled water, 24 hours' exposure to copper in dilutions up to 1 : 1,000,000 was fatal. In 1 : 10,000,000 dilution the organisms were still living, tho reduced in

number, after 2 weeks. Our water strain of B. coli, therefore, shows no such marked resistance as was reported by Clark and Gage.[3]

Moore[11] made the statement that B. typhosus was even more easily killed than B. coli, on exposure to copper. Table 7 gives the results of experiments with B. typhosus. In tap water this organism, like B. coli, continued alive in dilutions of 1:10,000,000 after 2 weeks' exposure. In a higher concentration, 96 hours' exposure killed. In twice-distilled water, all dilutions, even 1:10,000,000, killed in 24 hours. The difference in susceptibility between our strains of these organisms is therefore very slight.

TABLE 7

Results of the Action of Copper on B. Typhosus in Twice-distilled Water (glass), and in Sterile Tap Water

Time of Exposure		CuCl₂				
		0.1%	0.01%	0.001%	0.0001%	0.00001%
In twice distilled water	1 hr.	0	1	2	115	226,920
	3 hr.	0	0	0.5	3	798
	24 hr.	0	0	0	0	0.5
	48 hr.	0	0	1	1	0
	96 hr.	0	0	0	0	0
	1 wk.	0	0	0	0	0
	2 wk.	0	0	0	0	0
In sterile tap water	1 hr.	0	2	2,509	44,445	1,170,870
	3 hr.	0	18	76	272	249,118
	24 hr.	0	0	6	3	5
	48 hr.	0	0	3	4	45
	96 hr.	0	0	0	0	101
	1 wk.	0	0	0	0	36
	2 wk.	0	0	0	0	43

CONCLUSIONS

In the short time of an ordinary laboratory experiment and with the small amount of material usually employed, copper is unreliable and unsatisfactory both as a bactericide and as a fungicide. One percent frequently fails to kill all organisms within 15 minutes. As a rule, the degree of sterilization is proportional to the concentration of the copper and to the time of exposure. Only slight differences can be observed between the chlorid and the sulfate, this fact suggesting that such germicidal action as is present is dependent on the copper ion.

A certain specificity is apparent, some organisms (notably B. prodigiosus) being markedly susceptible to the effect of copper, while others (as Staphylococcus aureus and the moulds) are very resistant, surviving 15 minutes' exposure to even 5% solution.

As is to be expected, it usually requires lower concentrations for inhibition of growth than for killing, tho B. prodigiosus and to some extent B. coli seem to form an exception to this rule.

Our experiments indicate that in the purification of contaminated water supplies, with no limit as to time and with usually relatively few organisms distributed through a large amount of water, copper performs its function as a germicide much more efficiently than in the short-time experiment. Whether in closed sterile flasks or in large open vessels exposed to air and sunlight, it causes a diminution in the number of the ordinary water organisms and kills such bacteria as B. coli and B. typhosus (the most common pathogenic contaminators of water supplies) when in concentration of 1 part of the metal to 1,000,000 parts of the water—a concentration which could have no

TABLE   7—*Continued*
RESULTS OF THE ACTION OF COPPER ON B. TYPHOSUS IN TWICE-DISTILLED WATER (GLASS), AND IN STERILE TAP WATER

CuSO₄					Control
0.1%	0.01%	0.001%	0.0001%	0.00001%	
0	0.3	0.6	10,364	961,370	3,929,420
0	0.8	0.5	355	10,487	2,768,854
0	0	0.3	0	2	3,082,320
0	0	0	1	1	3,021,840
0	0	0	0	0	1,392,720
0	0	0	0	0	1,158,572
0	0	0	0	0	97,280
0	17	779	935	1,140,578	1,855,917
0	17	32	69	1,049,368	1,647,189
0	2	0.05	24	344,146	1,742,329
0	2	29	23	167,253	1,919,152
0	0	0.6	0	163,118	1,413,774
0	0	0	0	117,813	2,437,054
0	0	0	0	3,336	934,776

injurious effect on those using the water. To obtain this concentration about 2.5 parts of copper chlorid to 1,000,000 of water and about 4 parts of copper sulfate to 1,000,000 of water must be used. The amount used, however, must be varied in accordance with the amount of organic matter present and with the degree of contamination of the water. For this reason copper salts are far less efficient in the purification of sewage.

The fact demonstrated that very low concentrations of copper will, if sufficient time is allowed, kill or inhibit the growth of many pathogenic organisms, would seem to suggest that copper salts, since their toxicity is low, should have a certain therapeutic value, especially in diseases caused by those bacteria which are easily killed or inhibited by the metal. Its prophylactic and therapeutic use in typhoid fever and cholera has been noted in the literature cited. It has but slight bactericidal action on the tubercle bacillus, but in the treatment of an infec-

tious disease an inhibitory action may be, and probably is, quite as important as complete destruction. As already noted, a dilution of 1 : 100,000 prevents the growth of tubercle bacilli in the test tube. If, then, this concentration could be attained and maintained in the animal body, we might be able to prevent the further development of the disease and eventually to kill the organisms. However, the experiments of Corper[28] with various copper salts, and of DeWitt[26] with copper trypan blue, seem to show that soluble copper salts, when introduced into the animal body, are changed into insoluble form and held for the most part at the point of injection, where they give rise to severe ulcerations and necrosis. If these salts are introduced by mouth, they are relatively innocuous; but Corper[23] found that, whether fed or injected, the salts used by him and also colloidal copper had no effect on the course of the tuberculous process; he was also unable to find any appreciable amounts of copper in the tuberculous glands or eyes. In other words, he was unable to show any specific affinity of copper for the tuberculous tissues.

AN EPIDEMIC OF APPENDICITIS AND PAROTITIS PROBABLY DUE TO STEPTOCOCCI CONTAINED IN DAIRY PRODUCTS*

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The occurrence of appendicitis in epidemic form, its seasonal prevalence, and its occurrence in several members of the same family have been noted repeatedly (Mantle,[1] Hood,[2] Martin,[3] Wahle,[4] Haim[5] and Rostouzew[6]).

The epidemic of appendicitis and parotitis studied by us occurred at the Culver Military Academy, Culver, Ind. The study was made at the request of the superintendent of the academy, Lieut.-Col. L. R. Gignilliat, to whom we wish to express our appreciation for the opportunity and also to Dr. C. E. Reed for much aid in the work. From February 21 to March 5, 1915, a period of 12 days, there occurred 8 cases of acute appendicitis ; 2 cadets developing appendicitis on the same day. Seven of the 8 patients were operated on and the diagnosis verified. All recovered. Only 7 cases developed during the rest of the school year—2 in October, 1 in January, 3 in April, and 1 in May.

Two cadets developed parotitis previous to the sudden outbreak of appendicitis ; 5 during the prevalence of appendicitis ; 27 during March and April, and 3 in May, making a total of 34 cases. From April 10 to 18 there occurred an average of 1 new case a day and on April 25 3 students developed the disease. During the epidemic of parotitis 3 cadets developed appendicitis. The parotitis was not limited to the cadets, whose ages ranged from 15 to 19 years, but occurred in several older individuals as well.

The occurrence, the character, the duration, and the complications of the parotitis were typical. The epidemics occurred without associated tonsillitis. The cases of appendicitis were so distributed among the population as to rule out the factor of trauma from violent exercise.

[1] Lancet, 1910, 2, p. 57.
[2] Ibid., 1, p. 1645.
[3] München. med. Wchnschr., 1912, 59, p. 2005.
[4] Ibid., p. 1438.
[5] Arch. f. klin. Chir., 1907, 82, p. 360.
[6] Mitt. a d. Grenzgeb. d. Med. u. Chir., 1906, 15, p. 564.

The total number of persons at the academy who ate in a common dining room of the same food was approximately 500. Of these, 430 were cadets, ranging in age from 15 to 19 years; 30 were members of the faculty; 40 were helpers, having to do with the preparation and serving of the food, etc. The sanitary conditions, the refrigerating plant, the kitchen and dining-room, the quality of food served, the method of handling food, the mode of life as to exercise, etc., and the general physique and health of the cadets were all found to be excellent.

The dairy products consumed were obtained from 4 independent sources. The milk and cream and the ice cream served at the mess were from a number of dairies directly under the supervision of the authorities at the academy. These dairies were kept in first-class condition, and the milk was cooled soon after milking, and refrigerated continuously until used. The butter, of first-grade quality, was an Indiana product from a neighboring town. The cheese was from southern Wisconsin. The ice cream served to many cadets at a neighboring shop was found to be prepared from unpasteurized cream obtained from local dairies under unsanitary surroundings and with deficient refrigeration.

Since virulent streptococci have been isolated frequently from the udders of cows with mastitis, and even from normal udders in model dairies (Rosenow[9]), and since epidemics of septic sore throat, scarlet fever, and typhoid fever have been traced to milk, the possibility that the outbreaks under consideration were due to bacteria in the dairy products had to be considered. Cultures and animal inoculations were therefore made of the milk, cream, butter, and cheese which were for general consumption at the mess, and of the ice cream which was consumed by the cadets at the neighboring shop. A similar study was also made of cultures obtained from the tonsils of cadets, of members of the corps who prepared and served the food, of members of the faculty, and of those that developed appendicitis.

Technic

The technic of making cultures and animal inoculations was similar to that described in connection with the production of appendicitis.[7] Material for the cultures in the cases of parotitis was obtained from Steno's duct of the involved gland by catheterization and from the tonsils. An attempt was made to obtain material from the depth of the tonsils and not merely by swabbing the surface. The sediment of from 50 to 200 c.c. of milk and cream was used for the inoculations of the media. The butter and the cheese

[7] Jour. Infect. Dis., 1915, 16, p. 240.

(approximately 0.5 c.c.), obtained in a sterile manner from the depth of a freshly cut surface of the original package, were emulsified in 2 c.c. of NaCl solution and then planted.

The bacteriologic study consisted in the main of making blood-agar-plate cultures and inoculations of the material to be examined into a series of tall columns of ascites (10%) dextrose (0.2%) broth. These were incubated at 37 C. over night; then the character of the growth on the plates was noted and smears of the cultures in broth were made; those cultures in which no bacilli or only a few were found, were used for intravenous injection into rabbits and dogs. The bacteria for these injections were suspended in salt solution so that 1 c.c. contained the growth from 15 c.c. of the broth culture. A portion of the suspension injected was again plated on blood agar. The animals, often injected in series with doses ranging from 1 to 6 c.c., were chloroformed usually in 48 hours, if they had not already died from the effects of the injection. The examinations were made as soon after death as possible. The organism responsible for the lesions was determined by culture and from sections.

RESULTS OF INVESTIGATION

Sections from 4 of the human appendices, all showed streptococci, some in almost pure growth. In the case of one patient cultures were made both from the tonsils and from the wall of the appendix. That from the tonsils showed a predominating number of short-chained, green-producing streptococci, a few hemolytic streptococci, and a moderate number of Micrococcus catarrhalis; those from the wall of the appendix showed colon bacilli and green-producing streptococci. The culture in ascites dextrose broth from the tonsils of this patient was injected into 3 rabbits. All remained well and none showed lesions in the appendix. The growth from the appendix wall produced lesions in the appendix in 2 of 4 rabbits.

The findings in the case of another patient further illustrate the results obtained:

M., a cadet who developed symptoms of acute appendicitis on February 21, was operated upon on the following day and the acutely inflamed and edematous appendix removed. The lumen of this appendix was found to be very narrow and filled with bloody pus. There was no fecal concretion or other foreign body, and there were no constricting bands. The peritoneal coat was edematous and opaque and over the portion near the distal end was a thin fibrinous exudate. The mucous membrane was edematous and hemorrhagic throughout the larger portion, this condition extending well into the submucosa and the peritoneal coat. Sections showed an enormous number of streptococci within the lumen and within the infiltrated membrane (Figs. 1 and 2). Scattered diplococci were found also in the adjacent lymph follicles and in the peritoneal coat. In the lumen there were also a few gram-negative bacilli resembling colon bacilli, and what appeared to be fusiform bacilli. Cultures from a swab of the tonsils sent me by Dr. Reed 10 days after the operation, showed a predominating number of green-producing streptococci, a few colo-
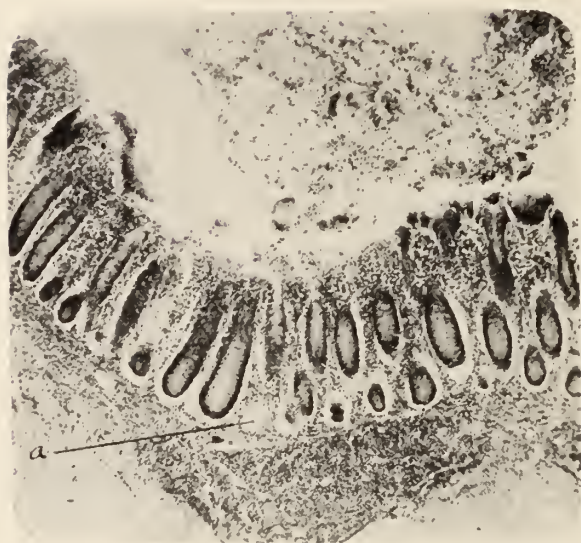
Fig. 1. Section of the appendix in human appendicitis. Note the sloughing of the mucous membrane, the hemorrhagic and leukocytic infiltration in the lymph follicle, the mucous membrane, and the submucosa. Hematoxylin and eosin. × 75.
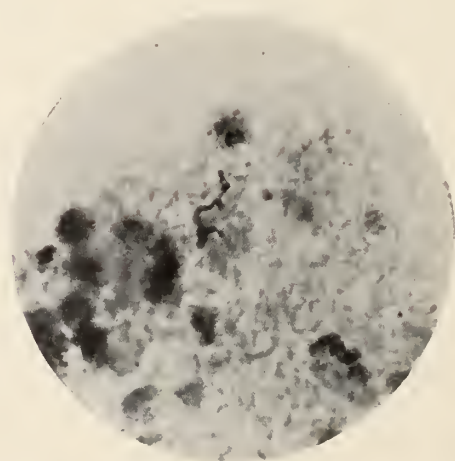
Fig. 2. Streptococci in submucosa at *a*, shown in Fig. 1. Gram-Weigert. × 1200.

nies of hemolyzing streptococci, and a large number of colonies of Micrococcus catarrhalis. The broth culture revealed a pure growth of a short-chained streptococcus. Two rabbits were injected with the latter culture, one of which showed hyperemia and hemorrhages in the mucous membrane and the peritoneal coat of the appendix. It also showed a few hemorrhages in the tricuspid valve. The other rabbit showed only a slightly turbid joint fluid. Cultures from the blood of both on blood-agar plates disclosed pure growths of green-producing streptococci. The emulsion of one of the areas of hemorrhage in the peritoneal coat of the appendix showed many green colonies of streptococci.

On June 4 cultures from the tonsils were made again. The tonsils were larger than normal but not badly infected. The culture in ascites dextrose broth was injected into one rabbit; it developed a number of small hemorrhages in the appendix with hyperemia and edema, as well as a marked hemorrhagic edema of the parotid and associated lymph glands. There were also a number of hemorrhages in the muscles, particularly in the adductors of the thighs. The localization in the parotid is of interest, especially since this individual was the janitor in the hospital in which the patients with parotitis were treated and hence may be considered a possible carrier.

TABLE   1

Localization of Streptococci Following Intravenous Injection

Sources of Streptococci		Time of Experiments	Animals	Strains	Percentage of Animals Showing Lesions in	
					Appendix	Parotid
Tonsils	Normal individuals	Soon after epidemic of appendicitis (March)	49	43	30	10
		Soon after epidemic of parotitis (June)	30	30	6	20
	Individuals that had had appendicitis	Soon after epidemic of appendicitis (March)	19	4	47	0
Dairy products..................		Soon after epidemic of appendicitis (March)	22	9	41	9
		During epidemic of parotitis (March and April)	28	8	0	29
		Soon after epidemic of parotitis (June)	10	6	0	30
Steno's duct in parotitis........		During epidemic of parotitis (March and April)	19	9	15	73

Table 1 gives a summary of the results following intravenous injection of streptococci isolated from tonsils, dairy products, and Steno's ducts. It is seen that the cultures obtained from the tonsils of normal individuals soon after the epidemic of appendicitis and at the beginning of the epidemic of parotitis produced lesions of the appendix in 30%, and in the parotid gland in 10% of the animals injected. After the epidemic of parotitis, cultures made in exactly the same way produced lesions in the appendix in 6% and in the parotid in 20% of the animals injected. The lesions in the appendix here correspond with the average

incidence of lesions in the appendix (5%) following injection of stepto-cocci from a wide range of sources.[8]   The cultures made soon after the epidemic of appendicitis from the tonsils in individuals who had had appendicitis produced this disease in 47% of the animals injected and no lesions in the parotid. Strains isolated from the dairy products soon after the epidemic of appendicitis, including cultures up to March 19, produced lesions in the appendix in 41%, and in the parotid in 9% of the animals injected; while during and soon after the epidemic of parotitis the strains failed to produce appendicitis, but produced parotitis in 29 and 30%, respectively, of the animals injected. The strepto-cocci obtained from patients having parotitis during the epidemic produced lesions in the appendix in 15%, and in the parotid in 73% of the animals injected.

In this connection it should be noted that 4 of the 6 individuals whose tonsils were cultured and who had to do with the serving of food, including the waiter at the table at which 2 cadets developed appendicitis on the same day, showed streptococci having affinity for the appendix of rabbits. One of them is subject to repeated mild attacks of appendicitis.

The average incidence of lesions in the various organs and the rate of mortality in animals injected with cultures from the tonsils or the throats of 46 normal individuals during March was 14 and 41%, respectively; while during June they were only 8 and 33%, respectively. The evidence of infection in the tonsils was distinctly greater during March, altho none of the individuals complained of sore throat and in none was the inflammation acute. The occurrence of lesions was more frequent in the animals injected with cultures from distinctly infected tonsils (18%) than in animals injected with cultures from more normal tonsils, or normal throats (4) in which there had been a previous ton-sillectomy (10%). Likewise, there was a distinctly higher incidence of lesions and a greater rate of mortality following injection of streptococci from the dairy products during March and April (11 and 26%, respectively) than during June (8 and 10%, respectively). These findings suggest that the seasonal prevalence of streptococcal throat infections is largely due to an increase in infective power of the streptococci in tonsils and possibly in dairy products as well.

It must not be supposed that the lesions in the appendix and the parotid are merely accidental. Cultures from the milk and the cream produced lesions in the appendix on March 6 and 19 while those

[8] Rosenow: Jour. Am. Med. Assn., 1915, 65, p. 1687.

injected on March 13, April 25, and June 4 failed entirely to produce lesions. In only one rabbit did the cultures from the milk and the cream produce parotitis (March 12). Cultures from the butter, made on March 5 and 13 and on April 9 and 25 and June 4 did not produce lesions in the appendix in rabbits, but did produce lesions in the parotid. Cultures from samples of butter obtained on April 9 showed a large number of 2 types of colonies of streptococci : the one produced distinct green colonies on blood agar ; the other produced smaller, grayish, elevated, round colonies surrounded by a narrow hazy zone of hemolysis. Injections of the mixture produced marked edema and hemorrhage of the parotid in 2 rabbits. Pure cultures from the latter (second culture) produced marked edema and hemorrhage of the parotid in 2 of 3 rabbits. The strain which grew in pure culture in dextrose broth produced similar lesions in 2 rabbits, and after injection into Steno's duct in each of 3 dogs produced the swelling and cellular infiltration considered characteristic of mumps. (The results of the experiments with the organism from parotitis, which closely resembled the organism described by Herb,[9] will be detailed in a separate paper.) The cultures from the cream and the ice cream supplied at the neighboring shop produced marked lesions of the appendix in 2 rabbits injected March 19, while cultures on two subsequent occasions after the plant was remodeled failed to produce either appendicitis or parotitis. Cultures from the cheese failed to produce either appendicitis or parotitis on March 15, but produced appendicitis on March 13, and parotitis on April 25 and on June 4. Cultures made May 7 and June 4 from butter and ice cream from another source failed to produce either appendicitis or parotitis. Investigation showed that in the communities where the butter and the cheese were manufactured mumps was present in epidemic form during April and May—the time during which parotitis was so prevalent at the academy and during which there were found in the butter and the cheese streptococci having such marked affinity for the parotid gland. The possibility of infection of the butter and the cheese with these strains at the academy is excluded because the cultures were made from the original packages ; but whether these strains were from human sources or were from the udders of cows it is impossible to say. It might be said, however, that the fermentative powers of some of the strains having affinity for the appendix suggest the latter origin, while those of strains producing the parotitis suggest the former source.

[9] Arch. Int. Med., 1909, 4, p. 201.

The cultures from the dairy products showed a preponderance of non-hemolyzing, short-chain-producing streptococci, often in almost pure form and in enormous numbers. Slightly hemolyzing streptococci were found occasionally. All were of a relatively low grade of virulence, but those producing parotitis caused death more frequently than did those producing appendicitis.

The importance of the streptococci contained in the dairy products as a possible source of infection for man is further shown by the fact that 6% of the animals injected showed ulcer of the stomach, 6% cholecystitis, 28% arthritis, 6% endocarditis, 20% myocarditis, and 26% myositis. The high incidence of myositis and myocarditis, which occurred 2 and 5 times as often as they occur with streptococci from the tonsils, is of special interest and in accord with the findings of Rosenow and Moon,[10] who showed that streptococci from milk during an epidemic of sore throat had a marked affinity for the muscles in animals.

The lesions other than those in the appendix and the parotid following injection of streptococci from the tonsils parallel very closely indeed those obtained previously by one of us[11] with streptococci from similar sources. It is realized of course that in man liability to infection following ingestion of the streptococci is less than it is in animals following intravenous injection. Yet, that infection may occur must be conceded.

The normal individuals during (10%), and immediately after (20%), the epidemic who harbored streptococci having elective affinity for the parotid in animals, must be regarded as carriers.

It would appear, then, that these epidemics of appendicitis and parotitis were due to streptococci contained in dairy products. This fact and the fact that milk is such an excellent culture medium make efficient pasteurization, or some other means of destroying the pathogenic bacteria which may be present in milk, imperative in order that the public health may be safeguarded.

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[10] Jour. Infect. Dis., 1915, 17, p. 69.
[11] Rosenow: Ibid., 1912, 11, p. 338.

THE PRODUCTION AND COLLECTION OF B. COLI IN QUANTITY ON SYNTHETIC MEDIA *

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The literature on synthetic media for the cultivation of bacteria was consulted with the view of procuring the best means of obtaining B. coli in quantity for a careful study of the chemical composition and the products of metabolism of this organism. To meet the requirements of the work as desired, the media ultimately selected should give a luxurious growth, the bacterial crops should be easily separable from the substrate by a simple mechanical operation which would give an uncontaminated product, and the medium itself should be in a condition that would warrant careful examination for substances occurring therein by virtue of the bacterial growth. Such a medium must obviously contain known and simple constituents in accurately measured quantities and would best be of the consistency of stiff jelly.

Experimental work on the growth of B. coli with a protein-free medium does not appear, as far as the author is aware, before the experiments of Galimard and Lacomme.[1] These workers used amido-acids as the source of nitrogen and combined these with salts. Their best medium had the following composition: leucin 7.5 gm.; alanin 2 gm.; tyrosin traces; glycerin 15 gm.; sodium chlorid 0.5 gm.; magnesium sulfate 0.05 gm.; calcium glycerophosphate 0.2 gm.; and distilled water 1 liter. This medium, which is neutralized with sodium carbonate, has no doubt a fixed chemical composition, but it is very complex.

In 1908 Dolt[2] succeeded in growing B. coli in several liquid and solid synthetic media. He used mainly asparagin as his amido-compound and found that a 1% solution, together with 0.2% of disodium or diammonium phosphate, made neutral to phenolphthalein with sodium hydroxid, gave a very satisfactory medium for the cultivation of B. coli. For a solid medium he added 1.5 % of purified agar. However, in trying to obtain a medium which would inhibit the growth of other water organisms, Dolt used ammonium lactate, malic acid, and glycerin in place of asparagin, and found that these gave better results. Therefore he especially recommended the following formulas for two media: for the one, purified agar 1.5%, glycerin 1%, and diammonium phosphate 0.2%; and for the other medium, purified agar 1.5%, ammo-

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* Received for publication November 3, 1915.

[1] Jour. de physiol. et path. gén., 1907, 9, p. 481.

[2] Jour. Infect. Dis., 1908, 5, p. 616.

nium lactate 1%, and disodium phosphate 0.2%. Both these media should be neutralized with sodium hydroxid and treated with 1% lactose before sterilization.

EXPERIMENTS WITH MEDIA

These media were tested in this laboratory, various combinations of the constituents and of the quantities of each under several conditions being tried. The following are illustrative of the general results obtained, and among them one was sufficiently satisfactory to serve the purposes.

Medium A: Three grams of agar were dissolved in 100 c.c. of distilled water. To this were added 1 gm. of glycerin and 0.2 gm. of diammonium phosphate. After the solution had been heated for 10 minutes on the water bath, 1 gm. of lactose was dissolved in it.

Medium B: Three grams of agar were dissolved in 100 c.c. of distilled water and 1 gm. of ammonium lactate and 0.2 gm. of disodium phosphate added. This mixture was heated for 10 minutes as in Medium A and then 1 gm. of lactose was added.

Medium C: A solution of 3 gm. of agar, 1 gm. of asparagin, and 0.2 gm. of disodium phosphate was prepared.

Medium D: This was prepared similar to Medium C with the exception of a substitution of diammonium phosphate for the sodium salt.

Medium E: Three grams of agar were dissolved in 100 c.c. of water and 1 gm. of calcium lactate and 0.2 gm. of diammonium phosphate were added.

Medium F: Two grams of agar were dissolved in 100 c.c. of water; 2 gm. of glucose, 0.1 gm. of diammonium phosphate, 0.5 gm. of magnesium sulfate, and 1 gm. of calcium carbonate were added.

Medium G: A repetition of the preparation of Medium B but without the addition of lactose.

Medium H: Three grams of agar, 1 gm. of Witte's peptone, 0.5 gm. of sodium chlorid and 0.5 gm. of meat extract were dissolved in 100 c.c. of water.

In all these media, purified powdered agar was used. After solution had taken place, the mixtures were filtered into incubating flasks, in which they were sterilized in a Bramhall-Dean autoclave. Each flask was streaked with 2 loops of a 24-hour broth culture of B. coli [communis] and incubated at 37 C. Observations were made at the end of 24 and 48 hours.

Dolt's glycerin medium (A) gave only a slight growth, as did also the calcium lactate mixture (E). Most of the other protein-free media (B, C, D, and F at 24 hr.) gave a medium growth, while the ammonium lactate without the lactose addition (G) gave an excellent crop, even better than the peptone medium (H). The growth in Medium G was examined microscopically as well as culturally and found to be pure B. coli.

The substitution of diammonium, for disodium, phosphate in Medium G gave almost the same results. Dolt's glycerin medium without lactose gave a good growth but not nearly so abundant as that of Medium G. Experiments using ammonium tartrate with disodium or diammonium phosphate, and in combination with ammonium lactate, gave only fair results in the first two cases and a result equal to that from Medium G in the last case. Medium G was therefore selected as a proper and most profitable medium for the cultivation of B. coli in quantity.

METHOD OF CULTIVATION AND COLLECTION

Nicolle and Alilaire[3] have described a pan suitable for growing bacteria in quantity which, after several minor modifications, has been adopted for the work of this laboratory. Our pans (manufactured for this laboratory by J. Pontius, Geneva, N. Y.) are made of copper, tinned on the inside. Their dimensions, selected to suit our sterilizing and incubating apparatus, are 36 cm. in length, 28.5 cm. in width, and 4.5 cm. in depth. The lower pan is surrounded by a small gutter, or groove, into which the cover is fitted. This gutter catches the excess water of condensation which forms during incubation. The cover is in the form of a large dihedral angle and is provided with an extra lip which overlaps the gutter on all sides, thereby making the chances of contamination considerably less (Figs. 1, 2, and 3). The condensation on the cover is further provided against by fastening Chardin paper on the inner side by means of strips of tinned copper, which fit into suitable flanges provided to hold the strips firmly in place. Eight of these pans were utilized for each harvest, giving a total agar surface of about 7400 sq. cm.

A little difficulty was experienced at first in making up the medium. It was found that if the sterilization was carried out too long, the ammonium lactate was liable to decompose. The filtration of such a stiff agar in a short time also presented a problem. After several modifications in technic, the following was adhered to almost exclusively.

One hundred grams of shred agar and 8 gm. of disodium phosphate were dissolved in 4 liters of distilled water by heating in the autoclave. (The constituents may be divided into 2 portions and dissolved in 2 large Erlenmeyer flasks.) When completely in solution, the mixture

[3] Ann. de l'Inst. Pasteur, 1909, 23, p. 547.

was filtered through glass wool and the reaction corrected to $+ 0.3$ (this reaction giving a most satisfactory final acidity). The filtration in this manner was very rapid. Five hundred cubic centimeters were then poured into each of 8 one-liter Erlenmeyer flasks (narrow mouth) each of which contained 5 gm. calcium carbonate. This addition of

Fig. 1. Front view of the cover.
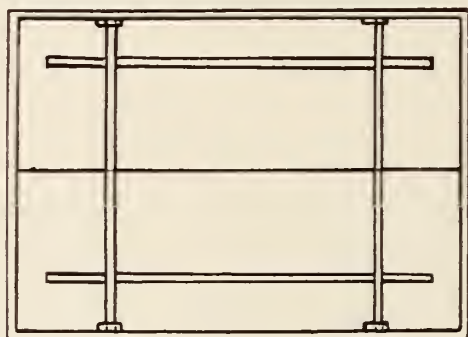
Fig. 2. Inside view of the cover (one-half the size of the other figures).
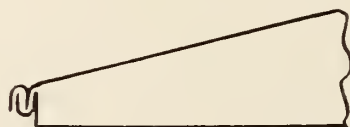
Fig. 3. Cross-section of the gutter.

carbonate is not absolutely essential and can be omitted if desired. Five grams of ammonium lactate were then added to each flask with shaking. After plugging, the flasks were sterilized in the autoclave for 15 minutes at 12 pounds pressure.

The pans which had been previously sterilized (the Chardin paper can be replaced by paper toweling, which is much cheaper and fully as efficient) were set on a level table and the media, after thorough

shaking, transferred to them with all aseptic precautions. The
cover of the pan was raised only on one side and just enough to receive
the mouth of the flask. Control experiments showed that contamina-
tion was rare in this transference. After a lapse of 2 hours, the pans
were cool and ready for inoculation. - This was accomplished with a
DeVilbiss atomizer in preference to swabbing, as better results were
obtained with the former method in several trial experiments. For
inoculation, a 24-hour culture in ordinary broth of B. coli [communis],
isolated from human feces, was utilized. The culture was carefully
transferred to a sterile atomizer and sprayed onto the surface of the
medium by lifting the cover of the pan only a very little distance, like
the opening of a hinge. The DeVilbiss atomizer has many advantages
in that the nose can be pointed in any direction one desires to spray
and can also be flamed, as it is constructed of metal. The air, which
is filtered through a glass bulb filled with cotton or glass wool, draws the
culture from the atomizer by suction instead of pressure. An essential
advantage of this method is the introduction of such a very small
amount of inoculating culture. Inasmuch as a protein-free medium is
being used, it is highly desirable to introduce as little of the peptone
medium as possible, and this is best overcome by the atomizer. Con-
tamination took place only rarely by this method, and the growth was
uniform and distributed all over the surface of the pan, altho only 5 c.c.
were used for inoculation of about 930 sq. cm. of surface. The pans
were then placed in the incubator and kept at 37 C. for from 9 to 10
days.

Many methods were tried for the collection of the growth. Leach[4]
employed a glass rod with some sterile water and drew off the sus-
pension thus formed by suction. Nicolle and Alilaire[3] used pieces
of sterile cardboard handled with forceps. Neither of these methods
seemed satisfactory to the author for obtaining a maximal yield, so
recourse was had to an original procedure. A safety razor blade was
improvised with a brass rod handle, which was soldered on. The blade
was flamed and then carefully scraped across the medium at a definite
slant so as not to break the surface of the agar. With a little practice,
practically the entire growth can be removed without a trace of adher-
ing media. The organisms were then dropped into 75% alcohol
(4 parts of 95% alcohol to 1 part of water), and, after disintegration
of any lumps, allowed to stand for 24 hours. In several experiments it

[4] Jour. Biol. Chem., 1906, 1, p. 463.

was found that if a stronger alcohol was used, the cells tended to coagu-
late into inconvenient masses which might be a source of error. On the
other hand, a more dilute alcohol gave considerable trouble by greatly
retarding the filtration. The cells were filtered off by suction on a hard
paper (C. S. & S. 975), washed once or twice with 95% alcohol, and
dried in vacuo over sulfuric acid for 48 hours. The average yield
obtained from 4 liters of medium, or 8 pans, was from 4.5 to 5 gm. of
the pure dried cells.

CONCLUSIONS

A medium containing 2.5% agar, 1% ammonium lactate, and 0.2%
disodium phosphate, with or without 1% calcium carbonate, was found
to be the most satisfactory protein-free medium for the cultivation of
B. coli. The absence of lactose greatly increased the efficiency of the
culture medium.

Inoculation of the media is best executed with a DeVilbiss atomizer,
for the organism is spread evenly over the entire surface with the
smallest amount of culture, and the chances of contamination are
reduced to a minimum.

In the collection of the bacteria the best results are obtained by
scraping the surface of the agar with a sterile safety-razor blade and
dropping the growth into 75% alcohol.

The pans herein described are highly recommended for the cultiva-
tion of bacteria in quantity, as they may be employed to advantage in
any laboratory without much cost.

THE PRODUCTION OF A HYPERIMMUNE SERUM FOR INFECTIOUS ABORTION IN MARES *

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In carrying on investigations of infectious abortion in mares, it occurred to us that a prophylactic serum as a protective agent against the disease might be produced. The question arose whether Bacillus abortivo-equinus† would produce a sufficient degree of immunity in a mare for the serum to have any prophylactic properties, and whether the amount of this serum necessary for protection would not be too great for practical purposes.

An old mare was selected for the production of this serum. The animal was in good health, of medium size, and had never had any disease so far as we were able to discover. This mare had been used previously in an experiment to determine the effect of inoculation with B. abortivo-equinus. This had no effect on the production of a potent serum. The entire treatment was as follows:

Oct. 22, 1913.—The mare received by way of the jugular vein 0.25 c.c. of a 24-hour mixed-broth culture of B. abortivo-equinus, diluted in 5 c.c. of normal sterile salt solution. The temperature at the time of inoculation, 5 p. m., was 99.8; on October 23, at 9 a. m., it was 99.4.

Nov. 4.—The mare received 1.5 c.c. of culture as before. The temperature before inoculation, at 3 p. m., was 99.6. After inoculation, at 3:30 p. m., it was 99.6; at 4 p. m., 99.8; at 4:30 p. m., 100.4; at 5 p. m., 100.6; on November 5, at 9 a. m., 100.2; at 1 p. m., 102.8; and at 4 p. m., 103.4.

Nov. 12.—The mare received 3 c.c. of a 45-hour culture in 7 c.c. normal sterile salt solution. The temperature at 2:30 p. m., before inoculation, was 100. After inoculation, at 3:30 p. m., it was 101.2; at 4:30 p. m., 103.4; on November 13, at 8:30 a. m., 102.2; at 2:30 p. m., 103; on November 14, at 11 a. m., 101.6; on November 15, at 9 a. m., 99.6.

March 13, 1914.—The blood showed an agglutination titer of 1:250.

† Our attention has been called to the fact that in previous articles the name suggested by us for the organism causing infectious abortion in mares, Bacillus abortivus equinus, is a trinomial designation contrary to the accepted rule for bacterial nomenclature. This being the case, we should suggest the name, Bacillus abortivo-equinus. The trinomial name appears in "Investigations of the Etiology of Infectious Abortion of Mares and Jennets in Kentucky," by Edwin S. Good and Lamert S. Corbett, The Journal of Infectious Diseases, 1913, 13, p. 53; in "Bacillus Abortivus Equinus as an Etiological Factor in Infectious Arthritis of Colts," by Edwin S. Good and Wallace V. Smith, The Journal of Infectious Diseases, 1914, 15, p. 347; and in "Maintenance of the Virulence of Bacillus Abortivus Equinus," by the same authors, The Journal of Medical Research, 1916, 33, p. 493.

March 23.—A mixed vaccine, which had been washed from agar slants, heated to 65 C. for 2 hours, and carbolated 0.2 of 1%, and which contained approximately 12,560,000,000 bacteria per cubic centimeter, was injected intrajugularly. The vaccine, 2 c.c., was diluted in 3 c.c. normal sterile salt solution. The temperature rose 1 degree as a result of this injection.

April 6.—Mare received 5 c.c. as before. Temperature rose only 0.6 degree.

April 19.—Eight cubic centimeters were given. Temperature rose 1 degree.

May 4.—Ten cubic centimeters were given. Temperature rose 1.5 degrees.

May 19.—Blood was drawn for an immune serum; the agglutination titer of this serum was 1 : 10,000.

March 6, 1915.—A series of inoculations was started, the intention being to produce a hyperimmune serum to be used in combating infectious abortion of mares. Blood was drawn; its agglutination titer was 1 : 100. A mixed 48-hour broth culture was given, 0.25 c.c. intrajugularly. The culture was diluted with 5 c.c. salt solution.

March 15.—Received 1 c.c. as before.

March 23.—Received 2 c.c. of a 24-hour culture in 3 c.c. salt solution.

March 31.—Received 4 c.c. as before (24-hour culture).

April 8.—Received 5 c.c.

April 16.—Received 7 c.c. The animal seemed depressed and rather emaciated. It did not seem advisable to give any more of the culture.

April 24.—The animal appeared to be in much better condition. Blood to the amount of 500 c.c. was drawn from the jugular.

April 26.—Blood to the amount of 800 c.c. was drawn from the jugular. Both these lots were allowed to clot. The agglutination titer of these samples was 1 : 1250. This serum was labeled "4/26".

May 15.—Mare received 7.5 c.c. culture as before.

May 26.—Blood to the amount of 800 c.c. was drawn from the jugular and defibrinated. The whole defibrinated blood was used in tests. It was labeled "5/26".

In order to determine whether this serum was of any bactericidal value, it was put to the following test,[1] the sample labeled "5/26" being used.

One cubic centimeter of the mare's blood and 1 c.c. of normal horse serum were inactivated by heating to 56 C. for one-half hour. They were then diluted 1 : 50. Two cubic centimeters normal fresh horse serum were then diluted 1 : 10. A 24-hour broth culture of B. abortivo-equinus was diluted 1 : 500.

One cubic centimeter of sterile broth was placed in 12 test tubes. To the 1st one of these, 1 c.c. of the diluted immune blood was added and thoroughly mixed. Of this mixture 1 c.c. was placed in the 2nd tube, 1 c.c. from the 2nd in the 3rd, and so on until the last tube was reached, from which 1 c.c. was discarded. The tubes now contained the following dilutions: 1 : 100; 1 : 200; 1 : 400; 1 : 800; 1 : 1600; 1 : 3200; 1 : 6400; 1 : 12,800; 1 : 25,600; 1 : 51,200; 1 : 102,400; 1 : 204,800.

Four tubes with normal horse serum were treated as the 12 tubes just mentioned. The dilutions in these tubes were: 1 : 100; 1 : 200; 1 : 400, and 1 : 800. Of the diluted bacterial emulsion 0.5 c.c., and of the diluted complement serum 0.5 c.c. were added to each tube.

[1] Kolmer: Infection, Immunity and Specific Therapy, p. 349.

Table 1 gives the results of the test for bactericidal activity on the part of the immune serum (A), together with the results of 8 control tests (B).

TABLE 1

A.—RESULTS OF BACTERIOLYTIC TEST OF IMMUNE BLOOD "5/26"

Tube	Dilution of Immune Blood	Mixed Bacillus abortivo-equinus, 24-hour Culture	Horse Complement	Results*
1	1: 100	0.5 c.c.	0.5 c.c.	Thousands of colonies†
2	1: 200	0.5 c.c.	0.5 c.c.	Sterile
3	1: 400	0.5 c.c.	0.5 c.c.	Several hundreds
4	1: 800	0.5 c.c.	0.5 c.c.	Several hundreds
5	1: 1600	0.5 c.c.	0.5 c.c.	About 200
6	1: 3200	0.5 c.c.	0.5 c.c.	About 250
7	1: 6400	0.5 c.c.	0.5 c.c.	About 100
8	1: 12,000	0.5 c.c.	0.5 c.c.	Less than 100
9	1: 25,600	0.5 c.c.	0.5 c.c.	Thousands
10	1: 51,200	0.5 c.c.	0.5 c.c.	Many thousands
11	1: 102,400	0.5 c.c.	0.5 c.c.	Infinite numbers
12	1: 204,800	0.5 c.c.	0.5 c.c.	Infinite numbers

B.—RESULTS OF THE CONTROL TESTS

Control	Purpose of Test	Substance Tested	Substances Used in Test	Results
1	To show the number of colonies in culture	0.5 c.c. culture	10 c.c. agar cooled to 42 C.	Infinite numbers
2	To show that the number of colonies had multiplied during incubation	0.5 c.c. culture	1.5 c.c. broth‡	Colonies denser than in Control 1
3	To show bacteriolytic properties of complement	Tube 1. 1 c.c. complement, 1:10		Many thousands
		Tube 2. 0.5 c.c. complement, 1:10	0.5 c.c. culture and broth enough to make 2 c.c. added to each tube	Infinite numbers
		Tube 3. 0.2 c.c. complement, 1:10		Infinite numbers
		Tube 4. 0.1 c.c. complement, 1:10		Infinite numbers
4	To show sterility of complement	0.5 c.c. complement	1.5 c.c. broth	No colonies
5	To show sterility of hyperimmune blood	1 c.c. immune blood 1:100	1 c.c. broth	No colonies
6	To show sterility of control serum	1 c.c. control serum 1:100	1 c.c. broth	No colonies
7	To show possible presence of complement in immune blood	1 c.c. immune blood 1:100	0.5 c.c. culture and 0.5 c.c. broth	Infinite numbers
8	To show possible presence of complement in control serum	1 c.c. control serum 1:100	0.5 c.c. culture and 0.5 c.c. broth	Infinite numbers

* In each of 4 tubes prepared with normal horse serum, instead of the immune serum, the number of colonies was infinite.

† In bactericidal experiments, the paradoxic results obtained, as in this instance, were caused by excess of amboceptors in the immune serum. Kolmer states (p. 354):[1] "In a mixture of bacteria, complements, and large amounts of amboceptor, the complement is bound not only by the amboceptors anchored to the bacteria, but also in a large measure by free amboceptors that are not anchored to bacteria. A portion of the anchored amboceptor, therefore, finds no complement at its disposal, and is therefore unable to exert any bactericidal action which gives rise to a relative lack of complement."

‡ The last 7 controls were incubated for 3 hours. All the tubes were then plated in agar, the plates incubated at 37 C. for 24 hours, and then the colonies counted.

Altho the plate in which the dilution of the immune blood was 1 : 200 was the only plate absolutely sterile, yet the bacteriolytic power of the blood was very marked in the dilutions of 1 : 400, 1 : 800, 1 : 1600, 1 : 3200, 1 : 6400, and 1 : 12,800. We consider that since the number of colonies in the dilution of 1 : 12,800 was less than 100, the titer of the serum must lie somewhere near that dilution.

Since this blood exhibited such excellent bacteriolytic power in vitro, experiments were made to see whether these effects could be shown in vivo.

In preliminary experiments, we determined that 1 c.c. of a 24-hour mixed-broth culture of B. abortivo-equinus subcutaneously administered, aborted a guinea-pig in 5 days, and that 0.1 c.c. culture given intravenously, was the lethal dose for rabbits in from 3 to 4 days.

On April 29, 1915, Rabbit 194 received 0.5 c.c. of a 24-hour mixed-broth culture of B. abortivo-equinus intravenously, and 3 c.c. of the hyperimmune serum "4/26" subcutaneously. Death occurred on May 5, 1915.

On April 29, Rabbit 192 received 0.5 c.c. of the culture intravenously, and 5 c.c. of the serum "4/26" subcutaneously. This animal developed marked symptoms, stopped eating, had decided conjunctivitis, but recovered and was normal again on May 17. A slight congestion occurred at the seat of inoculation on the margin of the ear, but this wound soon healed.

On April 29, Rabbit 199 received 0.2 c.c. of the culture intravenously, and 10 c.c. of the serum "4/26" subcutaneously. This animal died on May 3.

Five-tenths cubic centimeter of the culture represented 5 times the lethal dose. With the following animals this dose was cut to 0.1 c.c., with more satisfactory results, altho 0.1 c.c. had previously been shown to be extremely pathogenic, killing in from 3 to 4 days.

Rabbit 178 received 0.1 c.c. of a 24-hour mixed-broth culture intravenously, and 3 c.c. of the hyperimmune serum "4/26" subcutaneously. This rabbit developed no dangerous symptoms and became normal in 10 days. On the 12th day 0.5 c.c. of the culture was given intravenously. This caused rather severe symptoms, but the animal was completely recovered in 5 days.

Rabbit 176 received 0.1 c.c. of the culture and 5 c.c. of the serum. This rabbit developed no symptoms at all. On the 12th day it was given 0.5 c.c. of the culture intravenously. This secondary dose also produced no ill effects, the rabbit being completely normal in 3 days. It had gained 43 grams in weight during the experiment.

Rabbit 179 received 0.1 c.c. of the culture and 10 c.c. of the serum. As no symptoms were produced, the rabbit, 12 days later, received 0.5 c.c. of the culture. No ill effects were produced.

A pregnant guinea-pig received 1 c.c. of a 24-hour mixed-broth culture of B. abortivo-equinus subcutaneously, and 5 c.c. of the hyperimmune blood "5/26" also subcutaneously. This guinea-pig aborted 4 fetuses 17 days after the inoculation—12 days longer than is usually required for an abortion after such a dose.

From these experiments, it looked as if the hyperimmune serum were producing marked effects, and the following test was made on a pregnant mare.

A preliminary blood test showed that the mare had no immunity to the disease. The animal received, intrajugularly, 1 c.c. of a mixed culture washed from an agar slant and diluted in 5 c.c. of normal salt solution, and 200 c.c. of the hyperimmune blood "5/26," subcutaneously. For the first 2½ days after inoculation the animal did not eat. After this no symptoms were observed, except that the temperature rose to 104 F. 2 days after inoculation. On the 13th day, a well-developed fetus was discharged and B. abortivo equinus was recovered from its organs. The hyperimmune serum had not protected from an aborting dose of the organism.

Among the questions now being studied in this laboratory are (1) whether a larger dose of the serum will protect; (2) whether the serum will prove of value in a natural infection; (3) whether the serum can be produced in sufficient quantities to prove of practical value; (4) whether a vaccine will prove of value in building up sufficient immunity to protect against the disease.

SUMMARY

A hyperimmune serum for infectious abortion in mares can be produced that has marked bacteriolytic properties in vitro.

This serum protects rabbits from the lethal dose of the organism. In one instance it protected from 5 times the lethal dose.

The serum lengthened the time for abortion in one guinea-pig 12 days.

The serum did not protect a mare from an artificial infection. The amount of the organism given, inoculated directly into the blood, was vastly more than could be gotten in a natural infection. The serum may prove of value in a stud where the infection is known to exist.

STUDIES ON INTRADERMAL SENSITIZATION, I [*]

INTRADERMAL REACTIONS TO EMULSIONS OF NORMAL AND PATHOLOGIC SKIN

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The present studies were undertaken as extensions of a clinical investigation of an obscure dermatosis (pityriasis lichenoides chronica). A review of the phenomena of cutaneous sensitization, in this connection, led to an extension of the experiments within a limited field, and to an effort—based in part on these experiments and in part on reported advances in the literature—to correlate and explain numerous conflicting observations. Inasmuch as these seemed to have arisen to some extent from efforts to apply clinically the older immunologic principles, the author undertook to re-interpret such clinical tests as the luetin and the pallidin tests for syphilis (Noguchi,[1] Klausner[2]) and the cutaneous test for pregnancy (Engelhorn and Wintz[3]) based on the original conception of the Abderhalden reaction in this condition, and his own results, in the light of recent work on the physical mechanism of anaphylaxis. The author regards his own work as incomplete, and the generalizations as a working theory the applicability and usefulness of which are yet to be fully determined.

HISTORICAL REVIEW

The parenteral introduction into the body of proteins of homologous organ extracts has been carried out by numerous investigators for nearly every organ of the body. The use of such extracts in intradermal and other cutaneous tests is also familiar. Sellei[4] reported a group of experiments on psoriatics in which he believed that evidence had been secured of an allergic condition of the skin in this disease. By subcutaneous injection into 8 psoriatics, of a specially prepared emulsion from psoriatic lesions, he obtained, he asserts, both local and systemic reactions, including in some cases rises of temperature and extension of the eruption. Control injections of normal-skin emulsion were negative.[5] Bruck,[6] in a discussion of specific skin hypersusceptibility to iodoform, antipyrin, tuberculin, etc., referred to Sellei's observations with the sug-

[*] Received for publication November 10, 1915.

[1] Jour. Exper. Med., 1911, 14, p. 557.

[2] Arch. f. Dermat. u. Syph., 1914, 120, p. 444.

[3] München. med. Wchnschr., 1914, 61, p. 689.

[4] Wien. klin. Wchnschr., 1909, 22, pp. 1183, 1216.

[5] Berl. klin. Wchnschr., 1910, 47, p. 1836.

[6] Ibid., p. 517.

gestion that an attempt be made passively to sensitize guinea-pigs against psoriatic-skin emulsions by means of injections of psoriatic serum. In a second report on the behavior of skin emulsions of the type employed in his earlier experiments, Sellei[5] described what he believed to be a selective hypersusceptibility of the normal skin toward its own proteins. This was evidenced by a more marked reaction on injection of a skin emulsion into the person from whom the skin was taken, than could be obtained with the same emulsion in another normal person. A similar effect was claimed for prostatic secretion. This type of hypersusceptibility, Sellei designated as "homaesthesia."

Through the courtesy of Dr. Wm. Allen Pusey the author was given the opportunity to apply Sellei's methods to a case of pityriasis lichenoides chronica (parapsoriasis), and to follow up Bruck's suggestion of a possible passive transmission of this form of sensitization. Material from a case of psoriasis was also obtained through the courtesy of Dr. Udo J. Wile. The author and Mr. Vincent Kozilek then supplied the material for a re-examination of Sellei's conclusions regarding "homaesthesia." Finally, experiments with an intradermal agar reaction were begun, the results of which are reported also.

Preparation of Emulsions of Normal and Pathologic Skin

The directions given in Sellei's second paper[5] were followed in all essential details. No specification is given by him as to the use of local anesthesia, which the author employed, with precautions. Sellei's emulsions after preparation were incubated for 24 hours in his first series but not in his second, and mechanically shaken for 2 hours. In the experiments here reported, the emulsions were shaken for one-half hour without second incubation.

Under asepsis, after soap, alcohol, and ether cleansing, pieces of skin averaging 1.5 cm. by 3 cm. were excised under 2% cocain anesthesia, each annular wheal being well outside the area excised. Incisions extended through the cutis, avoiding as far as possible the subcutaneous fat. This tissue was minced in a sterile glass mortar with 3 c.c. of salt solution, and dried in the incubator for 24 hours. The material was then, under asepsis, ground up with 10 c.c. of salt solution containing 0.5% phenol. Two hours' grinding reduced all but a small residue of connective tissue. The suspension thus produced was so finely divided that it did not settle appreciably in 24 hours or more, and could be drawn through a 22 or 24 gage needle. After a vigorous shaking for at least one-half hour it was placed on ice. Centrifugation of such an emulsion at high speed yielded an opalescent fluid. The whole emulsion was used in the experiments. After 24 hours on ice the sterility of the suspension was tested by culturing on glucose and Loeffler's blood-serum agar. The few organisms inevitably introduced with the skin had died out within this time, leaving the material sterile. The sterility of the suspensions was subsequently tested occasionally and they were kept on ice in the dark.

Sellei gave no directions for the standardizing of his emulsions by the use of weighed amounts of skin, so that the solid content of various emulsions probably varied. While this must constitute a criticism on results involving comparisons, the emulsions intended for comparative use in the present case were made of approximately equal strength.

EXPERIMENTS IN PITYRIASIS LICHENOIDES CHRONICA

For this work a pityriasis-lesion emulsion (pityriasis lichenoides chronica) was made from two typical active lesions excised from the patient's left arm, no normal skin being included. Then a normal-skin emulsion was prepared from a piece of skin of approximately equal size, excised from the author's leg between the knee and the ankle (uniform precautions being taken in regard to local anesthesia, etc.). Finally, a psoriatic-lesion emulsion was obtained from a typical lesion in an untreated psoriatic (courtesy of Dr. Udo J. Wile). This emulsion was somewhat more concentrated than the other two, because of the size of the lesion excised. No normal skin was included. During shipment from Ann Arbor, this emulsion was kept at room temperature for 16 hours.

The serum used in these experiments was obtained from aseptically drawn blood, allowed to clot at room temperature for 15 minutes. Clot and red cells were separated by centrifugation at 3000 revolutions for 7 minutes, and the serum used within 45 minutes, without cooling. Microscopic examination disclosed an occasional leukocyte and a few scattered red cells.

EXPERIMENT 1.—Scarification tests with the emulsions were made, the scratches being deep enough to draw blood. The results were negative.

EXPERIMENT 2.—This duplicated the experiments by Sellei[4] on psoriatics with psoriatic-lesion emulsion. At the beginning of a period of improvement (regarded by Sellei as the most favorable time), 0.25 c.c. of the pityriasis-lesion emulsion were injected subcutaneously below the patient's left scapula. Altho there was some local sensitiveness, after injection, the marked local swelling and redness with rise of temperature and extension of the eruption described by Sellei for psoriatics were absent. A second injection 7 days after the first, with double the original dose, was negative. No signs of sensitization by the preliminary injection, such as lighting up of the original site of injection, appeared. Subsequent intradermal injections showed the emulsion to be active, so that deterioration could hardly have been a factor.

This failure to elicit evidence of an allergic reaction on the part of the patient to emulsion of his own lesions was followed by experiments on guinea-pigs, and it was not until later that intradermal injections were taken up. While the previous injection of emulsion subcutaneously, and the use of autogenous serum therapy, may conceivably obscure the interpretation of the results, the results are none the less of interest.

EXPERIMENT 3.—The pityriasis-lesion emulsion, 0.05 c.c., was injected intradermally into the skin of the arm below the deltoid insertion. An equal amount of normal-skin emulsion was similarly injected into the skin of the other arm. Of the two emulsions, that of normal skin, if any, contained the greater amount of suspended material. The course of the reaction was as follows:

1st Day.—Split-pea-sized, wheal-like papules at both sites.

3rd Day.—Well-marked reaction to the patient's own emulsion (pityriasis) : shotty red nodule, 8 mm. in diameter, elevated, with central high point, but no fluctuation or signs of pustulation. The reaction to the control injection of normal-skin emulsion was scarcely visible, barely palpable, and with the areola did not exceed 3 mm. in diameter.

4th Day.—Active nodule exhibiting a slightly translucent center, suggestive of fluid. Erythema subsiding. Control negative.

6th Day and Thereafter.—Involution in progress. Nodule persisted for 2 weeks or more. A translucent bluish center developed, which disappeared without rupture or crusting.

This reaction, at its height, was as definite as, for example, the usual moderately positive luetin reaction. It corresponded well to the papular type (Noguchi[1]), and its development and duration, as well as the obvious difference between it and the control of normal-skin emulsion, seem to entitle it to consideration as evidence of greater reactivity on the part of the patient's skin to emulsion of his own lesions.

EXPERIMENT 4.—On the 7th day after the intradermal injections of the preceding experiment, a second series of intradermal injections was made—the dose being 0.1 c.c.—with psoriatic-lesion emulsion, pityriasis-lesion emulsion, normal-skin emulsion, and the serum of the patient.

2nd Day.—Well-marked reaction to psoriatic emulsion and to patient's own emulsion, but practically none to emulsion of normal skin or to patient's serum. Rose-red areolae, 12 and 14 mm. in diameter, with hard central nodules. Both sites of reaction decidedly sensitive.

3rd Day.—Nodules more marked and shotty, areolae slightly smaller. Controls negative.

4th Day.—Nodules developing translucent centers as in previous experiment.

5th Day and Thereafter.—The erythema fading rapidly, leaving a dusky bluish-red nodule visible for 2 weeks and palpable for longer.

No lighting up of the previous sites of injection occurred. The response to psoriatic emulsion was a surprise, and was fully as distinct as the failure to respond to normal skin. At this time the patient's condition was stationary, just preceding a severe exacerbation. The skin was fairly clear. The last autogenous serum injection had been given 1 month before the first intradermal test series (Experiment 3). Normal-skin emulsion was apparently active in a series on normal skin 12 days after Experiment 4.

EXPERIMENT 5.—In the interval of 27 days from the beginning of Experiment 4 to that of Experiment 5, the patient had received potassium iodid, 45 gr. daily for 1 week, with no effect on the involuting nodules. The condition of the skin had undergone a marked change for the worse. Intradermal injections were made—dose 0.1 c.c. each—with a new emulsion of normal skin from Mr. Kozilek, pityriasis-lesion emulsion, psoriatic-lesion emulsion, and bismuth subnitrate, a 20% suspension in olive oil.

The results of this series seemed to indicate a diminished reactivity of the skin. There were no areolae as in previous experiments, and scarcely more than palpable nodules, which were well advanced towards involution by the 3rd day. In so far as a difference was apparent, the psoriatic-lesion emulsion failed to give a' reaction. The emulsions of normal skin and of the patient's skin caused slight reactions, about equal in degree. The bismuth suspension, which certainly should have been non-specific, produced a definite reaction but slightly less than that observed on normal skin. Judged by the usual

criteria, the reactions to skin emulsions were negative. The confirmation of the 3rd experiment by the 4th, in view of the fact that the normal-skin emulsion appeared from later tests to be active, seems to entitle the result to consideration.

EXPERIMENTS IN PASSIVE SENSITIZATION OF GUINEA-PIGS

While the studies described were in progress, an effort was made to transfer the problem to animals, as suggested by Bruck[6] for psoriasis. The technic employed was that of Bruck,[7] Friedberger and Ito,[8] Wolff-Eisner,[9] Klausner,[10] and others in the passive sensitization of guinea-pigs against tuberculin, iodoform, antipyrin, etc., by the injection of the serum of supposedly hypersensitive individuals. One subcutaneous, and one intraperitoneal series were undertaken.

SUBCUTANEOUS SERIES

Serum injected in the abdomen; emulsion injected 24 hours later in the shaved buttock.

Guinea-pig	Serum (5 c.c.)	Emulsion
1	Normal	Pityriasis-lesion 0.5 c.c.
2	Normal	Normal-skin 0.5 c.c.
3	Pityriasis	Normal-skin 0.5 c.c.
4	Pityriasis	Pityriasis-lesion 0.5 c.c.
5	Normal	Normal-skin 0.5 c.c.
6	Normal	Pityriasis-lesion 0.5 c.c.
7	Normal	Normal serum 5.0 c.c.
8	Normal	Pityriasis-lesion 5.0 c.c.
9	Normal	Normal serum

No immediate reactions occurred. Guinea-pigs 2 and 4, which had received skin and serum from the same individual, developed fever with infiltrates and sloughs at the sites of injection of the serum. Guinea-pigs 1 and 2 developed infiltrates but no sloughs. One of the serum controls also developed a local reaction. The effect in Guinea-pigs 2 and 4, while explainable by the injection of serum alone (Arthus and Breton,[11] Volk,[12] and others), so far suggested Sellei's "homaesthesia" that it was subsequently re-investigated with further controls.

INTRAPERITONEAL SERIES

Guinea-pigs, weighing approximately 500 grams each, were treated in accordance with the following schedule, second injections being given 24 hours after the first.

Guinea-pig	Serum (1 c.c.)	Emulsion (0.25 c.c.)
1	Normal	Normal-skin
2	Normal	Pityriasis-lesion
3	Normal	Pityriasis-lesion
4	Normal	Normal-skin
5	Pityriasis	Normal serum
6	Normal	Normal serum
7	Normal	Normal-skin
8	Normal	Normal-skin

Guinea-pig 6 (normal-serum control) died in 48 hours with convulsions and a fall of temperature to 92 F. The blood fluid, and the lungs were nega-

[7] Berl. klin. Wchnschr., 1910, 47, pp. 519, 1929.
[8] Ztschr. f. Immunitätsf., 1912, 12, p. 241.
[9] Dermat. Centralbl., 1907, 10, p. 164.
[10] München. med. Wchnschr., 1910, 57, p. 1451.
[11] Compt. rend. Soc. de biol., 1903, 55, p. 1478.
[12] Arch. f. Dermat. u. Syph., 1911, 109, p. 174.

tive. Guinea-pig 7 (normal-skin emulsion) died on the 14th day with progressive emaciation. Examination negative. While evidencing the toxicity of the serum and of the emulsions used, this method afforded no confirmation of the previously suggested "homaesthesia" or evidence of skin-serum interaction. A later subcutaneous series with fresh normal-skin emulsions, made as part of the work with normal skin, showed the reaction previously noted to be apparently indistinguishable from that produced by serum alone.

SUMMARY OF THE RESULTS ON PITYRIASIS LICHENOIDES CHRONICA

No marked constitutional and local reactions to subcutaneous injection of lesion emulsions, such as have been described for psoriasis, could be obtained in this case of pityriasis lichenoides chronica (parapsoriasis) by similar procedures.

Scarification tests with skin emulsions were negative.

Intradermal tests, however, seemed to show during a remission a moderate but definite reaction to emulsions of psoriatic and parapsoriatic skin lesions, not to normal-skin emulsions. During a subsequent exacerbation of the eruption, the reactivity of the skin to the emulsions was apparently greatly diminished, and it had lost its selective character. Previous use of autogenous serum, subcutaneous injection of patient's skin emulsions, and administration of potassium iodid affect the conclusiveness of these findings to an uncertain degree, if at all.

Passive sensitization of guinea-pigs to emulsion of the lesions by patient's serum cannot be accomplished by the subcutaneous or the intraperitoneal routes.

No conclusive evidence of interaction, if such exists, between blood serum and skin substrate, as represented in the emulsions used, could be elicited from the experiments made thus far on guinea-pigs.

EXPERIMENTS IN PSORIASIS

Sellei's[4] conclusions were now tested more directly with the available psoriatic-lesion emulsion, on 3 psoriatics.

CASE 1.—Girl, aged 22, with guttate and serpiginous psoriasis of 10 months' duration, untreated. Received 0.5 c.c. of the psoriatic-lesion emulsion subcutaneously over left scapula. This emulsion was 28 days old, and apparently active 2½ months later. No reaction followed except some local sensitiveness. Repetition of the injection with the same dose 7 days later gave no result except slight local sensitiveness.

CASE 2.—Woman, aged 36, with syphilis of 18 years' duration, Wassermann-positive. Psoriasis of at least 30 years' duration. Scalp, elbows, and knees showed typical patches, extending slightly at the time of observation. Two weeks before the experiment, the patient had received one injection of autogenous serum (of hypothetical interest only).

Aug. 6.—Normal-skin emulsion, 0.1 c.c., was given intradermally on anterior aspect of thigh after alcohol-ether sterilization.

1st Day.—Central dark-red papule, split-pea-sized, painful. Pale red areola, 2 cm. in diameter. Patient complained of feeling nauseated, was constipated, pulse 100, face flushed. Symptoms discounted since patient was subject to similar disturbances from constipation.

3rd Day.—Nodule more marked. Areola still present but less marked. Feeling well.

7th Day.—Nodule shotty, same size as on 3rd day, bluish-red, with areola somewhat smaller than on 3rd day but still well marked. Patient stated that since last seen, the papule had become much higher, but had subsided under hot compresses used to reduce it and to relieve discomfort.

The perfectly definite reaction thus produced slowly subsided, the bluish-red nodule persisting without an areola for at least 2 weeks more.

On the 7th day after the first intradermal injection the patient received a second intradermal test, coincidently with the giving of a second dose of autogenous serum. As noted, the reaction from the previous test was still well defined.

Aug. 13.—The psoriatic-lesion emulsion, 0.1 c.c., was given intradermally on the anterior aspect of the thigh opposite to that injected in the preceding test, and normal-skin emulsion, 0.15 c.c., intradermally, at least 4 inches from this injection. The larger dose was accidental, due to sticking of the syringe piston.

1st Day.—At site of the injection of the psoriatic-lesion emulsion, central red nodule, areola 2 cm. At that of normal-skin emulsion, nodule slightly larger, areola 2.75 cm. No constitutional symptoms.

3rd Day.—At site of the injection of the psoriatic-lesion emulsion, a relatively large deep-red central nodule, areola about 3.5 by 4 cm. At that of the injection of normal-skin emulsion, a brawny infiltrated slightly raised plaque, with angry red center; deep nodular infiltrate of the size of half a small hazel nut. Areola 6 cm. in diameter. Extremely sensitive.

5th Day..—Both sites showed central infiltrations with papule-formation of a purplish-red color suggesting ecchymoses, especially marked for the normal-skin emulsion. The purplish nodule proper in this case was now as large as a medium-sized pea, and imbedded in a zone of palpable infiltration. Areolae almost disappeared.

7th Day and Thereafter.—The bluish nodules persisted for several weeks, developing a faint ring of yellow, suggesting an involuting ecchymosis.

As compared with that from the previous injection of a normal-skin emulsion in equal dose, the reaction produced by the psoriatic-lesion emulsion was the more marked. The severity of both these reactions and that to the larger dose of normal-skin emulsion should be compared with the reactions in the following case in a non-syphilitic psoriatic.

CASE 3.—Girl, aged 21, with psoriasis of 7 years' duration, treated with x-ray 1 year before. Guttate and serpiginous lesions of wide distribution. No intercurrent ailments. Second of 2 autogenous serum injections 3 weeks before intradermal tests.

Aug. 6.—Psoriatic-lesion emulsion, 0.1 c.c., was given intradermally, on the anterior aspect of thigh after alcohol-ether sterilization. Autogenous serum, 65 c.c., same day, intramuscularly.

1st Day.—No wheal or areola. Slightly inflamed papule, with shotty infiltration, but nodule no larger than original injection wheal.

2nd Day.—Stationary.

7th Day.—Practically disappeared.

This reaction then was at most only slightly positive and strikingly less evident than that in the psoriatic with old syphilis. After a 2 weeks' interval the following tests were made.

Aug. 20.—Psoriatic-lesion emulsion, 0.1 c.c., and normal-skin emulsion, 0.15 c.c., both intradermally as before.

1st Day.—At site of the injection of the psoriatic-lesion emulsion, a reddish nodule with small faint areola 1 cm. in diameter. At that of the injection of normal-skin emulsion, a more marked nodule, areola 14 mm. No constitutional symptoms.

2nd Day.—For the psoriatic-lesion emulsion, a reddish nodule, practically no areola, diameter about 8 mm. For the normal-skin emulsion, well-defined central nodule, areola 2.5 cm.

3rd Day and Thereafter.—The reaction to psoriatic-lesion emulsion fading rapidly, scarcely characteristic. That to normal-skin emulsion fairly persistent but certainly not strikingly positive. In view of the larger dose the response was less than normal, judged from later experiments.

SUMMARY OF THE RESULTS IN PSORIASIS

No constitutional reaction or effect on the eruption was observed in any of the cases receiving injections. It is of interest here that many of Sellei's[4] reactions were obtained only on repeated injections and often some time after the initial injection. A 2-weeks' interval in one of these cases was without influence on the reaction.

The local reaction occurred in convincing form only on intradermal injection.

In Case 2 with equal doses of normal-skin and of psoriatic-lesion emulsion there seemed to be a somewhat more marked reaction in the case of the psoriatic. The difference was slight, less marked than that produced by an increase of one-half in dosage.

No lighting up of the old sites of injection or lesions, or other evidence of systemic allergy was observed.

The most striking observation was that of the marked difference between the reactions, under identical conditions, of a psoriatic with late syphilis and of one without complications. In the former, intradermal reactions identical with marked papular luetin reactions, both in appearance and duration, were obtained with normal-skin and psoriatic-lesion emulsions. In the latter, the reactions to identical doses under the same conditions were almost doubtfully positive— less positive even than was established by later work as normal for the normal-skin emulsion.

While the bearing of the last point on the luetin reaction itself will be further discussed, this finding deserves consideration as evidence of the non-specific character of intradermal reactions such as the luetin and pallidin reactions in late syphilis.

EXPERIMENTS WITH EMULSIONS OF NORMAL SKIN

For this work fresh suspensions of normal skin were prepared by the methods previously outlined. Both donors (the author and Mr. Vincent Kozilek) were normal individuals with negative histories as regards specific diseases and cutaneous conditions. The pieces excised were approximately equal in size (2 by 5 cm. before excision), from identical sites on the donors' legs between knee and ankle. The blood was expressed by pressure; the fat was scraped off.

An emulsion of blood clot was prepared to secure a homologous protein and ferment suspension other than skin, which might by its activity afford a clue to the part played by blood proteins and enzymes in the cutaneous reaction. The clot from 12 c.c. of blood was repeatedly washed with physiologic salt solution, centrifugated, dried, and prepared in the same way as the emulsions of skin.

Experiments were now begun to determine the character of the local and of the general reaction in those guinea-pigs of the previous subcutaneous series which had received serum and skin from the same individual. The doses in the series here discussed were proportioned on the basis of 1 c.c. serum and $\frac{1}{5}$ c.c. emulsion per 100 grams' bodyweight. Large guinea-pigs were used.

Two guinea-pigs, injected subcutaneously in the abdomen with, in the one, 8 c.c. author's serum and 1 c.c. author's skin emulsion; and in the other, 7 c c. author's serum, and $1\frac{1}{5}$ c.c. Kozilek skin emulsion, developed sloughs and infiltrates on the abdomen; but the first guinea-pig also had fever, lost weight, and died on the 20th day. The somewhat more marked reaction in the first guinea-pig was then controlled by the following series.

Guinea-pig	First Subcutaneous Injection	Second Subcutaneous Injection
3	Author's serum 5 c.c. in wheal, abdomen	Author's serum $\frac{5}{6}$ c.c. in back
4	Author's serum 5 c.c. in wheal, abdomen	....
5	....	Author's skin emulsion $\frac{5}{6}$ c.c. in abdomen
6	Author's serum 7 c.c. in wheal, back	Author's serum $\frac{7}{8}$ c.c. in abdomen
7	Author's serum 7 c.c. in wheal, back	Author's emulsion $\frac{7}{6}$ c.c. in abdomen
8	Author's serum 6 c.c. deep between muscles of abdomen	....
9	Author's serum 7 c.c. deep between muscles of abdomen	Author's serum $\frac{7}{8}$ c.c. in back
10	Author's skin emulsion $\frac{6}{8}$ c.c. in wheal, abdomen	Author's serum 6 c.c. in back
11	Author's serum 7 c.c. in back	....

The results with this series seemed to justify conclusions as follows: 1. Sloughs and fever can develop in guinea-pigs as a result of the injection of serum alone when this is injected subcutaneously in the abdomen (Guinea-pig 4). 2. Deep injection of the serum, even in the abdomen, tends to prevent the development of sloughs and fever. 3. Subcutaneous injection of the serum into the back make a slough less likely, altho not absolutely preventing it (Guinea-pig 9). 4. In guinea-pigs in which local reaction to the preliminary injection of serum has been avoided, no further distinctive reaction can be elicited by the subsequent injection of skin emulsion. 5. Preliminary injection of skin emulsion, followed by that of serum, gives no distinctive result. 6. The intradermal injection of emulsion in doses approximating 1 c.c., gives rise in the course of 2 or 3 days to a small circumscribed non-inflammatory infiltrate, which shrinks to a pea-sized shotty nodule that may persist for several weeks. No constitutional symptoms were observed.

These results are comparable to those reported by Arthus and Breton[11] in the case of rabbits sensitized to horse serum. From these results and from the results of the preceding experiments with guinea-pigs the author feels reasonably convinced that the reactions observed in the subcutaneous series were not due to an antigen-amboceptor reaction in the guinea-pig between skin emulsion and serum, but to the serum alone. Sellei's[4] views, therefore, are scarcely susceptible of demonstration on animals, at least by the methods used.

An incidental impression gained in the course of these experiments is in accord with that of Volk,[12] who in a critique of the work of Bruck,[7] Klausner,[10] and others on passively transmitted sensitization, called attention to the essentially gross character of the method. A large margin of experimental error must be allowed where the injury of an initial injection of a toxic substance such as foreign serum is followed by the insult of a second toxic injection. It would seem that only an immediate and striking reaction with typical findings deserves consideration, and that misinterpretations follow the attempt to construe nondescript later symptoms and even ultimate fatal issues.

EXPERIMENTS ON NORMAL SKIN WITH SKIN EMULSIONS

These experiments were a direct investigation of Sellei's[5] "homaesthesia"—that is, the production of a more marked cutaneous reaction by an individual's own skin emulsion than by that from another person. Normal-skin emulsions (the older emulsion of the author's skin,

a fresh skin emulsion, and the Kozilek skin emulsion) and blood-clot emulsion, were employed, as well as fresh serum from new donors.

EXPERIMENT 1.—Intradermal injections, after alcohol-ether sterilization, of normal-skin emulsions (Kozilek's and the author's), 7.5 cm. apart, on the outer side of Kozilek's arm below the deltoid. Dose 0.1 c.c. of each. In 24 hours well-marked nodules had developed, and the areolae had reached diameters of 4.5 cm. The active inflammatory symptoms then began to subside, leaving palpable red nodules of split-pea size, which remained active for more than a week and then gradually faded, without any hemorrhagic changes. The two reactions were practically identical, the slight difference in favor of Kozilek's skin emulsion in the early stages being due to a slightly larger dose.

EXPERIMENT 2.—Repetition of Experiment 1 on the author's arm, doses 0.1 c.c. of author's skin emulsion and 0.15 c.c. of Kozilek's skin emulsion. Sensitiveness developed about 10 hours after injection, with annular zones of erythema about the developing indurations. The reaction to Kozilek's skin emulsion was the more marked; within 36 hours this emulsion produced a considerable edema with a large areola (4 by 6 cm.) and considerable lameness and sensitiveness. The areola subsided leaving a well-marked shotty red nodule, still prominent on the 6th day (size of a split pea) but fading thereafter, altho palpable and visible as a shotty papule for at least 10 days more. The reaction to the homologous emulsion in smaller dose, while definite, was much less marked, and by the 6th day had practically disappeared, leaving a slight purplish induration.

EXPERIMENT 3.—In this experiment a comparison of the reactions to skin emulsion and to blood proteins was made, and a comparison of the reactions to intradermal and to deep subcutaneous injection. The experiment was begun 4 days after Experiment 2. Injections were made intradermally in the right arm of the author, of his own skin emulsion, of Kozilek's serum, of blood emulsion, of normal-skin emulsion, of Kozilek's skin emulsion, and of his own serum. The same injections were made in the subcutaneous tissues of the author's left arm. Neither serum control reacted. On intradermal injection the reaction to the blood emulsion was of shorter duration and the papule-formation less distinctive than was the reaction to the skin emulsions. Sensitiveness was less marked. Kozilek's, and the old normal-skin emulsion produced nodules which are still persistent, tho no longer inflammatory, 8 weeks after injection. The immediate symptoms in both were marked. The author's skin emulsion, injected higher on the arm where the skin was thicker and less mobile, produced a less striking, but persistent, reaction. The deep subcutaneous injection produced reactions which were less distinctive clinically, but which nevertheless resulted in the slow development of deep infiltrations.

EXPERIMENT 4.—A repetition of the intradermal injections of the preceding experiment, Kozilek's arm being used. The serum controls were discarded. The experiment was made 19 days after Kozilek's first and only injection of emulsion.

3rd Day.—Two conspicuous reactions to Kozilek's skin emulsion and to the blood emulsion.

5th Day.—The reactions to the blood emulsion and to the two normal-skin emulsions (both from the author) had largely subsided. The reaction to Kozilek's skin emulsion had become the characteristic red nodule.

10th Day.—All the reactions were palpable, but otherwise practically negative.

It was noted that the inflammatory reaction about the sites of the injection of blood emulsions corresponded in each case exactly to the bluish zone of diffusion of the injected material. This was suggestive of the circumscribed character of the field of reaction in all the foregoing injections.

Experiment 5.—A portion of Kozilek's normal-skin emulsion was centrifugated at 3000 revolutions for 30 minutes, and the supernatant, slightly opalescent fluid injected intradermally, with normal-skin emulsions (the author's and Kozilek's), dose 0.1 c.c., into sites on the upper arm of a person who had received no previous injections. The fluid from the centrifugated emulsion caused no reaction. The normal-skin emulsions (Kozilek's and author's) produced reactions of the usual papular type, the former being the more marked.

5th Day.—The inflammatory areola on the site of the injection of Kozilek's emulsion was still well defined, the nodule shotty, 5 mm. in diameter.

7th Day.—This papule had begun to scale; the areola became dusky.

10th Day.—It was a slightly elevated scaling papule. The reaction to the author's normal-skin emulsion had practically disappeared, leaving a bluish spot.

A gradual decrease in the efficiency of the emulsions was noted, apparently as a result of the deposition of suspended material on the sides of the bottles, which could not be dislodged by shaking.

In order to ascertain whether the normal-skin emulsions contained anything capable of fixing complement in a hemolytic cycle, a complement-fixation test with guinea-pig complement, rabbit amboceptor, and sheep corpuscles was made, with negative results.

summary of experiments with emulsions of normal skin

Normal skin reacts to intradermal injection of emulsions of skin by a local inflammatory reaction, followed by the development of a more or less persistent papular, or nodular, infiltrate at the site of injection. This reaction is comparable to the papular type of the luetin reaction (Noguchi), and a positive reaction may be judged by the same criteria as a positive reaction in the case of luetin.

The reactions show considerable variation, dependent probably on dose and depth of injection, and a possible difference in skin reactivity in different individuals.

No constant specific character could be established in normal persons for the response toward their own, as compared with that to others' skin emulsions, nor was there any evidence confirmatory of Sellei's[5] "homaesthesia"—which was so definite that experimental error in the form of slight differences in dose, concentration of emulsions, depth of injection, and other technical details, could be eliminated.

Quantitative results should only be based on standardized emulsions.

A suspension of proteins, etc., obtained from blood clot gave rise to a definite, but slightly more transient, reaction.

The fluid in which the protein suspension is made, as obtained by centrifugation, was inactive. The reaction apparently is produced, therefore, by the solid constituents. While this paper was in preparation, the author used the remainder of Kozilek's emulsion to test the activity of boiled emulsion. Three minutes' boiling darkened the color and destroyed the homogeneity of the suspension. Altho there was some difficulty in getting precipitate through the needle without an undue proportion of fluid, a reaction was produced which showed a small, but distinct, papule 5 mm. in diameter, slightly scaling on the 6th day. The initial reaction gave an areola 2.5 cm. in diameter. A single attempt with a deteriorated emulsion is hardly conclusive, but with due allowance the result seems suggestive.

The emulsion apparently possessed no antigenic properties in a hemolytic complement-fixation cycle.

There were no evidences of so-called active anaphylaxis to the proteins of the emulsion, such as constitutional symptoms, or lighting up of previous sites of injection on second injections (at intervals of from 4 to 19 days).

The attempt passively to sensitize guinea-pigs to emulsions of skin by means of serum from the donor of the emulsion or from another person, was unsuccessful by subcutaneous and intraperitoneal routes.

STUDIES ON INTRADERMAL SENSITIZATION, II *

AN INTRADERMAL REACTION TO AGAR AND AN INTERPRETATION OF INTRADERMAL REACTIONS

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AN INTRADERMAL REACTION TO AGAR

The author's studies on the reaction to agar, tho incomplete, are given discussion here because the observations thus far seem to afford a clue to the mechanism of intradermal reactions in general. The author undertook these experiments after a review of recent work on the physical theory of "anaphylatoxin"-formation. Certain aspects of these newer conceptions seemed to afford an explanation of the phenomena observed with emulsions of skin, as well as of the non-specific aspects of reactions to such substances as luetin, pallidin, and placentin. Of further interest was the recent announcement by Sherrick[1] that intradermally injected agar—among the best understood of the colloid antiferment-adsorbents, and, in 0.5% suspension, easily available for intradermal tests—gives rise to reactions on the administration of potassium iodid which are clinically indistinguishable from the luetin reaction and that an involuting luetin reaction can be revived by the same drug.

Technic

The materials used in the first experiments in this direction were as follows : (1) A 0.5% agar suspension in physiologic salt solution (after being autoclaved, cooled, and shaken, this gel became a viscous translucent fluid which could easily pass through a fine needle) ; (2) a 20% suspension of bismuth subnitrate in salt solution ; (3) a 20% suspension of bismuth subnitrate in olive oil ; (4) olive oil alone.

All these materials were thoroughly sterilized in the autoclave before use. Instead of bismuth subnitrate, kaolin, another well-known antiferment-adsorbent, might have been used. The difficulty of introducing an insoluble powder into the cutis in salt suspension was so considerable and the violence of the reaction in the 1 successful attempt in 5 was such that the method was given up, and the oil suspension used to provide a so-called "foreign-body" reaction as a control comparison. Oil alone was used both as a control and to provide an illustration of the behavior of a neutral fat intradermally injected. The difficulty of injecting a powder suspension probably explains

Sherrick's reported failure to secure a reaction from bismuth. The powder packs in the needle, and a small amount of salt solution can be filtered through, which raises a wheal but introduces very little bismuth.

EXPERIMENT 1

Intradermal injections were made in the left forearm (author's), after alcohol-ether sterilization, with 0.1 c.c. each of 0.5% agar, 20% bismuth subnitrate in olive oil, and of olive oil alone.

The oil alone produced practically no reaction, the slight areola disappearing in 36 hours. No papule formed.

Bismuth in oil, produced a marked reaction. On the 3rd day, a nodule had formed, the size of a marrow-fat pea. A factitial wheal could be produced on rubbing

4th, 5th, and 6th Days—Itching especially mornings, with wheal-formation on rubbing. On one occasion a wheal formed increasing the diameter of the papule from 0.7 cm. to 1.7 cm.

8th Day and Thereafter.—This symptom disappeared, but the nodule has persisted with little sign of subsiding up to the present time (6 weeks). Nothing similar had been observed in previous reactions.

The agar produced a small discrete nodule, the size of a bird shot, with a rather large faint areola by the end of the 2nd day.

4th Day.—The nodule had flattened and all erythema had disappeared.

9th Day.—The nodule was reappearing with a translucent center and a marked areola.

11th Day.—A pea-sized papule with a soft, fluid-containing purplish center and a 3 cm. areola. The lesion then evacuated spontaneously, discharging a grumous bloody fluid. Immediate healing took place.

EXPERIMENT 2

A repetition of Experiment 1, on Mr. Kozilek. Oil alone and bismuth in oil gave the same results as before, except that no factitial wheal developed.

Agar produced an early reaction, marked, with deep induration, and a papule 8 mm. in diameter. Areola, 4 by 6 cm., transient.

9th Day.—Nodule persistent. Reaction began to light up spontaneously and develop a new areola.

10th Day.—Papule 8 mm. in diameter, hemispherical, with translucent, fluid-containing center. Areola 3.5 by 2.5 cm.

12th Day.—Nodule had increased to 1.5 cm. in diameter, was bluish-red in color, with a fluctuating center; areola 5 by 7 cm. Sensitive. Hemorrhagic character suggested by play of colors at margin.

13th Day.—Photographed (Fig. 1). Areola subsiding, but papule larger. Aspirated under asepsis. In spite of this the lesion continued to increase in size.

16th Day and Thereafter.—Lesion had reached a diameter of 2.5 cm., a raised, flattened, fluctuating, bulla-like lesion, purplish, with an infiltrated yellowish border. Free incision was made through a considerable thickness of skin, whereupon a grumous brownish fluid exuded. Lesion healed, leaving a small scar and a discolored patch. Absence of pain and lymphangitis rather striking.

Microscopically, the aspirated material was a mixture of pus and blood, and was sterile in smear and culture on a variety of media.

EXPERIMENT 3

In order to see whether an active anaphylaxis to agar had been induced in the author by the 1st injection, a 2nd intradermal injection of an entirely fresh preparation of agar (0.5% in salt solution) was made in the other arm 22 days after the 1st injection and 11 days after the evacuation of the hemorrhagic lesion.

1st Day.—Prominent red nodule, 10 by 8 mm., firm, fairly sensitive, with an areola 4.5 by 6 cm.

3rd Day.—Areola gone; nodule same size as before, mildly erythematous and slightly sensitive. Site of the first injection negative.

Fig. 1. Intradermal reaction to 0.5% agar on 13th day. The small papule to the right is a reaction to bismuth subnitrate in oil suspension, which is slowly involuting.

5th Day.—Nodule becoming purplish and soft, sensitive.

7th Day.—Nodule slowly enlarging. Contained fluid.

8th Day.—Papule high and prominent, a hemorrhagic fluid-containing lesion with a small faint areola. Distinctly sensitive and infiltrated. Site of the first injection, negative.

9th Day.—Nodule photographed. It ruptured spontaneously in the evening, discharging a bloody pus.

While the 1st reaction might be described as tardive, the 2nd passed through its cycle in about the average time for an ordinary luetin reaction. Otherwise, there was nothing to suggest an allergy towards agar after the 1st injection.

RESULTS IN THE STUDY OF THE INTRADERMAL REACTION TO AGAR

Intradermal injection of 0.5% agar into 2 normal individuals who had not been taking potassium iodid, produced a papulo-pustular or hemorrhagic pustular reaction, the course of which in the cases observed was similar to familiar types of the reaction to luetin described in the literature.

The 1st reaction obtained in Experiment 1 corresponded closely with the torpid form of the reaction to luetin as described by Noguchi,[2] Jeanselme,[3] and others.

Fig. 2. Intradermal reaction to 0.5% agar, second reaction on 9th day. Hemorrhagic-pustular lesion just before rupture. The macule to the left is a recent scar.

The reaction obtained in Experiment 2 suggested a severe pustular reaction to luetin.

On 2nd injection of a fresh preparation of agar similar to the 1st, 22 days after the 1st injection, a 2nd typical papulo-pustular "luetin" reaction developed. Active anaphylaxis was not apparent either sys-

[2] Jour. Exper. Med., 1911, 14, p. 557.
[3] Bull. Soc. franc. de dermat. et de syph., 1914, 25, p. 27.

temically or at the site of previous injection. The only difference between the two reactions was in the somewhat more rapid course of the 2nd.

The final stage of the reaction as observed thus far, is perhaps more distinctly hemorrhagic than the ordinary reaction to luetin.

Intradermal injection of a neutral fat produced no significant response.

The reaction to an insoluble non-protein powder (bismuth subnitrate) suspended in oil, after passing through an active early stage with nodule- and wheal-formation, subsided after about 10 days to an indolent nodule, representing probably an irritation phenomenon. The lack of a definite cycle such as is found in the agar and skin reactions may be due to the difficulty experienced by the tissues in disposing of the substance. The earlier phenomena seem to require, for their explanation, more than the conception of a merely mechanical process of local injury.

DISCUSSION OF THE RESULTS

The interpretation of these results and the discussion of intradermal reactions such as those to luetin, pallidin, placental extracts, etc., here offered, are based on recently developed views of the physical mechanism of anaphylaxis, as outlined in the work of Besredka and Ströbel,[4] Bordet,[5] Ritz and Sachs,[6] Keysser and Wassermann,[7] Kopaczewski and Mutermilch,[8] Doerr,[9] Bordet and Zunz,[10] Fenyvessy and Freund,[11] Dold and collaborators,[12] Jobling and his co-workers,[13] and various protagonists of the humoral view represented by Friedberger and his collaborators.[14] Especially serviceable are the discussions by Doerr[9] (he covers the entire field) and the summaries of the literature in the articles by Jobling and his co-workers.

In recent years two rival conceptions of the mechanism of anaphylactic intoxication have been conspicuous. That espoused, notably, by Friedberger and his school,[14] maintained, in substance, that the fate

[4] Compt. rend. Soc. de biol., 1911, 71, p. 413.

[5] Ibid., 1913, 74, p. 225.

[6] Berl. klin. Wchnschr., 1911, 48, p. 987.

[7] Ztschr. f. Hyg. u. Infektionskrankh., 1911, 68, p. 535.

[8] Ztschr. f. Immunitätsf., 1914, 22, p. 539.

[9] Wien. klin. Wchnschr., 1912, 25, p. 331. Kolle and Wassermann, Handb. d. pathogenen Microorganismen, 1913, 2, p. 947.

[10] Ztschr. f. Immunitätsf., 1914, 22, p. 42; p. 49.

[11] Ibid., p. 59.

[12] Ztschr. f. Immunitätsf., 1912, 15, p. 171; 16, p. 475; 1913, 18, p. 207. Berl. klin. Wchnschr., 1912, 49, p. 2310.

[13] Jour. Exper. Med., (a) 1915, 21, p. 239; (b) 1914, 20, p. 37. (c) Arch. Int. Med., 1915, 15, p. 286.

[14] Ztschr. f. Immunitätsf., (a) 1913-14, 20, p. 405; (b) 1913, 17, p. 506; (c) 1912, 12, p. 241.

of foreign proteins introduced into the body parenterally is accomplished through the proteolytic activity of specific enzymes which in an antigen-amboceptor reaction liberate from the digested protein matrix (antigen), the toxic substances responsible for the symptoms. This view, substantially unmodified, is the one most familiarly applied in the interpretation of the clinical phenomenon of anaphylaxis as ordinarily conceived. Within the past 5 years, however, the work of Besredka and Ströbel,[4] Bordet,[5, 10] Ritz and Sachs,[6] Keysser and Wassermann,[7] Doerr,[9] Mutermilch and Kopaczewski,⁣ and numerous other investigators, has compelled considerable modification of the older conceptions. Besredka and Ströbel, and Bordet, by showing that the exposure of normal guinea-pig serum to the action of 0.5% agar in vitro caused it to assume toxic properties which were apparent on re-injection into the same animals, opened the way for the conception that the matrix of "anaphylatoxin" lies, not in the parenterally injected substance, but in the serum or cells of the injected animal. The objection of the Friedberger school that the toxic effect of agar-treated serum was due to the effect of the serum enzymes on the protein of the agar itself was recently met by Bordet and Zunz,[10] who showed that an efficient "anaphylatoxin" could be produced by the action of pararabine, a practically nitrogen-free agar derivative. The same effect was demonstrated for a non-nitrogenous colloid—sodium pectin—by Kopaczewski and Mutermilch.[8] Numerous other investigations in the meanwhile had shown that a variety of substances, such as coagulated albumins, killed bacteria, certain toxins, and even such inert substances as kaolin, fuller's earth, and barium sulfate are capable of initiating the formation of "anaphylatoxin" from guinea-pig serum in vitro.

The mechanism of the action of these substances has been a matter of controversy. As a result of the investigation of bacterial "anaphylatoxin," Friedberger[14] was led to modify his theory to provide that the antigen-amboceptor reaction preceded the formation of "anaphylatoxin" and was the specific part of the reaction, the "anaphylatoxin" produced being the same regardless of the antigen. Subsequent investigations further tended to show that both specific antigen and amboceptor were unnecessary (Doerr,[9] Jobling[13]). Certain investigators committed themselves rather definitely to an exclusively physical theory of colloid interaction (Doerr[9]). Other investigations have tended to substantiate the view, apparently, that the development of "anaphylatoxin" in serum, for example, occurs as a result of lytic ferments normally pres-

ent, but inhibited by the presence of antiferment.* Agar, starch, kaolin, etc., act by adsorbing the antiferment, thus inhibiting its action, and liberating or uncovering the ferment proper. It is the uncovered ferments which then split the serum proteins up into toxic products responsible for the symptoms. In other words, the matrix of the toxin is now thought to be, not in the parenterally introduced substance, but in the serum or other proteins of the animal. The recent work of Jobling[13b] and his associates has been interpreted as showing that the antitrypsin (antiferment) is an unsaturated lipoid and that its action may be inhibited not only by adsorbents such as agar but by saturation with iodin, for example. These authors claim clinical application for this view in the well-known action of iodids in promoting lysis of granulomatous tissue. The specificity of the Abderhalden reaction has also been attacked on experimental, as well as clinical, grounds (Jobling, Eggstein, and Petersen[13a]). Agar, starch, etc., when used as substrates, give positive reactions with guinea-pig serum (Plaut[15]). Peiper[16] and Friedemann and Schoenfeld[17] regularly obtained positive Abderhalden reactions by adding starch to serum. Jobling and his collaborators[13a] showed that the placental tissue used in the reaction was not digested, but became more resistant to the action of trypsin, as a result presumably of adsorption of antitrypsin. These authors conclude "that the (serum) proteases are not specific, the placental tissue being found most efficacious possibly because of purely mechanical factors (surface exposure), as is indicated by the wide range of clinical conditions in which the placental substrate gives positive results." The placental tissue, then, acts as an antiferment-adsorbent, and not as the antigen on which a specific ferment in the serum acts. The matrix, therefore, of the protein split products which give a positive reaction is in the serum and not the substrate, according to this view.

The application of the physical theory of anaphylaxis, as distinguished from the humoral or chemical view, to the mechanism of intradermal reactions, develops a number of interesting and suggestive pos-

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* Whether the effect of an anaphylatoxin-forming agent, such as agar, kaolin, etc., is accomplished through proteolysis by ferments or through changes in colloid equilibrium in the affected tissues, serum, etc., need not impair the validity of the arguments advanced in this study against specificity and in favor of a common non-specific mechanism for the reactions subsequently discussed. The essence of the author's contention is that the intradermal reactions considered are not specific antigen-amboceptor reactions. The details of the non-specific mechanism, on the other hand, must be considered as unsettled. The conception of the mechanism has been phrased here in terms of the ferment proteolysis of a non-specific matrix because this view is, for the time being, supported by accessible experimental investigations.

[15] München. med. Wchnschr., 1914, 61, p. 238.
[16] Deutsch. med. Wchnschr., 1914, 40, p. 1467.
[17] Berl. klin. Wchnschr., 1914, 51, p. 348.

sibilities, especially when it is used to account for discrepancies and inconsistencies in what were at first accepted as specific tests in the older sense—that is, tests for specific amboceptors by the injection of specific antigens. Altho it would not be justifiable in the present status of experimental knowledge to apply the physical theory to the exclusion of the chemical, and to deny flatly the participation of antigen, amboceptor, and complement in the reaction, the physical theory seems unusually well fitted to harmonize and explain a confusion which has not tended to diminish with increased clinical experience under the older conceptions.

THE REACTION TO AGAR

The reactions to the intradermal injection of agar, as described in the first part of this report, seem to be the simplest and most convenient starting point for the application of the physical theory, in view of the prominent place that agar has held in the experimental work on which this theory is grounded. To recall the basic facts, agar was among the first of the colloidal producers of anaphylatoxin to be recognized, attention having been called to its properties by Besredka and Ströbel[4] and Bordet.[5] Working with a colloidal gel of 0.5% agar in physiologic salt solution, Bordet showed that a 3-hour exposure to its action at 37 C. would render a previously non-toxic guinea-pig serum extremely toxic for guinea-pigs, the symptoms induced being those of anaphylactic shock. The contention that this effect was due to adsorption was criticized by Friedberger[14] on the ground that agar contained enough protein (11%, König) to account for its behavior on the score that it acted as a protein antigen. This contention seems finally to have been met by the work of Bordet and Zunz[10] with pararabine (nitrogen-free agar) and by the work of Mutermilch and Kopaczewski[8] on pectin, Keysser and Wassermann[7] on kaolin, Nathan[18] on starch, and similar studies. The behavior of agar in an Abderhalden test with guinea-pig serum,[15] which normally contains large amounts of protease, has further tended to establish its action as that of an antiferment-adsorbent, and to place it in the same group as kaolin, and the other inert agents which in anaphylatoxin-formation must play a physical rather than a chemical rôle.

The transference of in-vitro results in the case of such adsorbents to in-vivo conditions, is only in its beginning. In the case of small laboratory animals, with serum of high protease content, the results should

[18] Ztschr. f. Immunitätsf., 1913, 18, p. 636.

be more clear-cut than in man. There would seem to be no reason a priori, however, why such a physical property as adsorptive capacity should be suspended on the parenteral introduction of a substance into the blood, or even into a local focus in the skin. Friedberger and Tsuneoka[14b] have recently studied the effect of kaolin in vivo (intravenous injection). Coming from the opposing camp, Friedberger's admission in this connection seems highly significant—"the toxicity of kaolin is not to be explained as of mechanical origin. It depends rather on adsorption *in vivo* of certain substances from cells, essential to the life of the organism."

To construct, then, a scheme for the action of intradermally injected agar in accordance with the theory of its action as a physical process, it may be conceived possible that the part which it performs is simply that of withdrawing from either the blood serum or the lymph, or from the tissues into which it was injected, or from all these, the ferments which protect them from autolysis. Autolysis then occurring, anaphylatoxins would be formed in a focal necrosis as the starting point of a reaction.

The matrix of this anaphylatoxin seems more open to dispute than the idea of its formation. Blood serum, leukocytes, and the cutaneous tissues themselves may serve as matrices. Human serum, for example, does not contain the relatively large amounts of protease found in the laboratory animals, the proteases in man being largely in the leukocytes (Jobling). It is possible, however, that adsorption of antiferment at a given point causes a rise in protease content in human serum such as occurs in tuberculosis, carcinomatosis, and pregnancy. The fall in antitryptic titer of serum in patients whose antitrypsin is being inhibited by iodids (Jobling and Petersen[13c]), is in line with such a view to some extent. Even more applicable is the observation of Börnstein, Nast, and Nickau,[19] discussed later, that a non-specific lytic action is developed in the blood after luetin injections. Favoring the significance of the surrounding tissues in the reaction, is the much more definite picture obtained by intradermal injection as compared with subcutaneous injection in all the types of reactions considered in this report.

Fixation of the adsorbing agent at the site of injection has seemed to the author an essential feature of the mechanism, necessary to explain the focal character. The slow diffusion of many substances from points of intradermal injection is a common observation, and was especially apparent in the injections of washed blood clot, for example, in which

[19] Arch. f. Dermat. u. Syph., 1914, 120, p. 240.

the zone of reaction corresponded exactly to the discoloration from blood pigments. It is possible that this slower diffusion accounts for the more striking reaction to intradermal injection as compared with that to subcutaneous injection,—instead of an active participation of the dermal cells as matrix for the anaphylatoxin. In studies of insect bites[20] that show a slow development (black fly), the author has felt that a local fixation of the toxic agent was essential to the explanation of the clinical and pathologic mechanism of the reaction. Such a fixation would of course reach an extreme in the case of an insoluble substance, such as bismuth subnitrate or kaolin, and might be a factor in the violence of the early reaction. It is conceivable that the action of certain supposedly inert "foreign bodies" may, in its earlier stages, be due to a physical adsorbent effect heightened by the mechanical effectiveness of their fixation.

The time element in a reaction, while to some extent a function of the degree of fixation of the injected adsorbent, may be conceived as depending also on the active resistance offered by the living cells to the action of the liberated proteases. This resistance, whether on the part of tissue or blood cells (leukocytes), would take the form perhaps of a formation of an anti-enzyme to replace that adsorbed, as well as of a possible mechanical removal of the adsorbent by phagocytosis. If the resistance overbalances the action of the adsorbent, involution takes place. If on the other hand, proteases were liberated with special ease or in special abundance, or the adsorption were very efficient, as might be the case under pathologic conditions, focal necrosis and autolysis would get the upper hand and the reaction would progress to pustule- or abscess-formation with evacuation of adsorbent and products of the digestion. Finally, in the event of a balance of this sort being reached, it is conceivable that it might be disturbed by another injection in the immediate vicinity, or the introduction of another antiferment-inhibiting agent such as iodin through the blood. This might, as mentioned later, provide a rationale for Sherrick's observations on the lighting up of agar and starch injection sites on the administration of iodids.

The clinical course of the agar reaction strongly suggests the plausibility of such conceptions of its mechanism. The true papular reaction developing within from 24 to 48 hours after injection represents supposedly the active fight of the surrounding tissues, and perhaps leukocytes, to produce anti-enzymes to replace those adsorbed. This phase

[20] Jour. Cutan. Dis., 1914, 32, pp. 751, 830.

occupies several days, and is accompanied by all the signs of acute inflammation. At the center of the lesion, where the concentration of the adsorbent is highest and the formation of anaphylatoxin by the liberated proteases most active, necrosis occurs, accompanied by proteolysis. This proteolysis is a conspicuous feature of the reactions to agar thus far seen by the author, in their later stages. The rapidity with which in one case, for example, the subcutaneous tissues were literally dissolved into a sterile grumous hemorrhagic fluid, was highly suggestive of a powerful lytic ferment action. This autolysis was conspicuous after the inflammatory symptoms representing the defensive reaction of the tissues had largely subsided.

Reactions in which constitutional symptoms occur in conjunction with the local ones, while not thus far observed with agar, may be conceived as following the escape of locally formed anaphylatoxin from the reaction site into the blood, or as resulting from the lysis of serum proteins by proteases temporarily uncovered by the adsorption of a considerable amount of serum antiferment at the site of injection of the adsorbing agent. If it be correct to place the leukocytes first as antiferment-carriers in the human body, for example, their function in the leukocytic wall that surrounds such local foci is apparent. Their antiferments guard the body against a general uncovering of its own proteases, serving also to restore the balance destroyed by the local effect of an antiferment adsorbent.

Adsorption reactions of the type which that to agar represents in the scheme outlined here, are obviously non-specific. The body does not according to this view, react to agar as such, but to adsorption of antiferments. The symptoms, then, are not evidence of the specificity of the injected substance in an antigen-amboceptor reaction, but merely the result of allowing the body to digest its own proteins by adsorbing or inhibiting its antiferments. The only value of such reactions, therefore, from the clinical standpoint, would be to measure the enzyme balance or lability, and the amount and intensity of action of nonspecific proteases in the body. The establishment by this method of the existence of ferment instability, etc., in a given case may prove to have clinical diagnostic value, and afford invaluable information on the modus operandi of pathologic agents.

It may be argued that the agar reactions observed, represent a special case, and that they would not occur in persons who had not already subjected themselves to a series of intradermal injections such as those of skin emulsion, bismuth, and the like. The question of the

amount of variability possible in the reaction of normal skin, aside, it is scarcely to be expected that a reaction to agar will occur in all individuals, nor is it necessary that it should. If the reaction to agar is an expression of ferment balance or ferment activity in the serum or tissues, it will undergo wide variations in normal and pathologic conditions. It would, in fact, be an additional piece of evidence in favor of the wide applicability of the physical theory, to be able to show that normal individuals who do not ordinarily react, could prepare themselves for reaction to a non-specific substance such as agar by a series of tests on themselves with normal-skin emulsion. That the agents which cause reaction to skin emulsions, to palladin, to placental extracts, to luetin, etc., can likewise cause a non-specific but clinically similar reaction to an antiferment adsorbent would almost amount to a demonstration of the essential identity of the mechanisms and the consequent non-specificity of the clinical tests mentioned. The settlement of all these questions must of course await further study of the agar reaction, in normal and pathologic individuals.

THE MECHANISM OF THE LUETIN REACTION

While the luetin reaction seems several steps removed from the plainer case of the agar reaction, a close examination of experimental evidence bearing on it, gives ground for placing it, in large part at least, in the class of antiferment-adsorbent or anaphylatoxin reactions, and treating it as in the case of the reaction to agar, not as specific for syphilis, but as a measure, albeit perhaps a sensitive one, of the ferment-antiferment balance, and of the amount or intensity of action of non-specific proteases in the body of the syphilitic.

Of immediate interest in this connection is the recent report by Börnstein, Nast and Nickau,[19] previously mentioned, of a non-specific lytic reaction in the serum of certain syphilitics. This reaction was observed to develop after intradermal injections of luetin. In so far as such findings indicate a relation between non-specific ferment activity and the luetin reaction, they have direct application to the previous contentions. The parenteral injection of an antiferment-adsorbent, by lowering the antiferment content, would uncover serum proteases of a non-specific character, and this could explain the reaction observed by these authors. This effect has been observed by Jobling and Petersen[13c] in the inhibition of antitrypsin by iodin, as mentioned. They report, it will be recalled, the demonstration in vivo in the case of syphilitics receiving potassium iodid, of a definite lowering of anti-

tryptic activity in the blood. This should mean non-specific protease in small amounts in the blood. If luetin is an antiferment-inhibitor, it could conceivably uncover enough protease to account for the reaction observed by Börnstein, Nast and Nickau.[19]

Argument of this indirect type is supported by an examination of luetin itself from the standpoint of the physical conception of anaphylatoxin-formation. Noguchi's[2] original description of the preparation and ingredients is in substance as follows. Spirochaeta pallida from ascitic-agar cultures are mixed with those from ascitic-fluid cultures, the agar mass in the first case, filled with the organisms, being ground in a sterile mortar, and the fluid culture added. Luetin, therefore, when ready for use, contains agar, diluted with ascitic fluid to an undetermined degree, and the fragmented bodies of spirochetes. The further dilution with salt solution is made at the time of injection. A control suspension consists of the similarly prepared culture media, uninoculated.

In the light of this description, the following considerations present themselves : Both the control and the luetin proper contain at the outset one of the best-known antiferment adsorbents—agar. Luetin, in contradistinction from the control, contains in addition the fragmented bodies of Spirochaeta pallida.

The production of so-called bacterial anaphylatoxin, probably by a mechanism of adsorption (Doerr[9]), has been demonstrated for a variety of organisms, including forms as closely related to the Spirochaeta pallida as the spirochetes of chicken spirillosis and of Russian relapsing fever (Mutermilch, Dold and Aoki, quoted by Doerr). In 1913, Nakano,[21] working with pure cultures, reported the formation of bacterial anaphylatoxin from guinea-pig serum by Spirochaeta pallida in vitro. On the basis of this experimental evidence, it seems not unreasonable to suggest that the fragmented Spirochaeta pallida in luetin may be capable of anaphylatoxin-formation in vivo on intradermal injection, the effect to be added to that produced by the agar anaphylatoxin provided for by the presence of that substance in the preparation.

These considerations provide at once a not unacceptable physical explanation of the greater efficiency of luetin as compared with its control. Luetin contains two adsorbents instead of one. The presence of an active antitrypsin-adsorbent in the control (agar), moreover.

[21] Arch. f. Dermat. u. Syph., 1913, 116, p. 281.

explains such results as those of Boas and Ditlevsen,[22] who in a series of cases, using an authentic preparation, obtained in lues tertius and lues hereditaria nearly as many reactions of almost the same intensity from the control preparation as from the luetin itself. Other non-specific or doubtful results have been reported by Burnier,[23] Cederkreutz,[24] Kaliski,[26] Schmitter,[26] Jeanselme,[3] Joltrain,[27] Bruck,[28] and Baermann and Heinemann.[29] Jeanselme, for example, obtained largely torpid positives. The problem of securing a luetin which will cause a reaction in a syphilitic skin and not in a normal one, may be one of securing such a dilution or proportion in the ingredients as will serve as a measure of the ferment hyperactivity or lability rather than one of providing specific antigen for a specific amboceptor to react with. The obvious difficulty in striking such a mean without accurate knowledge of the amount of each ingredient necessary to produce a local ana-phylatoxin reaction, is apparent, and may well explain the erratic behavior of certain specimens of luetin, attested by workers. Moreover, the tendency to discard the control after a few injections, observed in several favorable reports, has perhaps prevented some of these considerations from standing out as clearly as they might.

Clinical resemblance between the agar reactions observed and the luetin reaction, has impressed the author, even in an experience with the former which is much too incomplete to establish the foregoing considerations. In the reactions seen thus far, the conventional type of marked luetin reaction and the tardive form, as judged by the criteria of Noguchi,[2] Benedek,[30] Jeanselme,[3] Boas[22] and others, have been simulated to a degree which, allowing for the difference in the concentration, etc., of the adsorbents, is highly suggestive. Such close resemblance argues somewhat of a common mechanism.

Mention should be made at this point of Sherrick's[1] observation that reactions to intradermally injected agar light up on ingestion of potassium iodid.

In his preliminary report, thus far the only account he has published, he mentions moderate transient reactions from intradermally injected agar, following doses of 0.07 c.c. of a "less than 1%" solution. He lays special stress on

[22] Arch. f. Dermat. u. Syph., 1913, 116, p. 852.
[23] Bull. Soc. franc. de dermat. et de syph., 1914, 25, p. 31.
[24] Finske läk.-sällsk., handl., Helsingfors, 1913, 1, p. 407.
[25] N. Y. Med. Jour., 1913, 98, p. 24.
[26] Jour. Cutan. Dis., 1913, 31, p. 549.
[27] Bull. Soc. franc. de dermat. et de syph., 1913, 24, p. 507.
[28] Versamml. deutsch. Naturforsch. u. Aertze in Wien, 1913.
[29] München. med. Wchnschr., 1913, 60, p. 1537.
[30] Ibid., p. 2033.

the lighting up of the reaction following the administration of potassium iodid, and it is conceivable that, using this drug, he obscured the outcome of what might have been reactions to agar in certain cases, resembling those in the author. His dosage, moreover, was distinctly lower than the author's.

The lighting up of both luetin and agar intradermal-injection sites on the administration of potassium iodid, as reported by the same author, is an interesting additional evidence of the essential similarity of the two reactions as to mechanism, and a further means of linking intradermal reactions with other ferment reactions elsewhere in the body. Jobling and Petersen[13c] in their work on iodin action on antitrypsin, already mentioned, quote Michaud and Wells and Hedonberg as having demonstrated an increased concentration of iodin in necrotic tissues, especially when softening is in process. The function of this iodin is supposed by Jobling and Petersen, to be that of an inhibitor of antitrypsin, an action which they have demonstrated on serum. The administration of iodids supposedly supplies iodin to inhibit antiferment action in a focal necrosis and autolysis ensues. The sites of the injection of agar and luetin are presumably focal necroses. If they are conceived as due to the action of ferment-inhibitors, the involution possibly represents the reaching of a local balance between increased amounts of protease and antiferment. As soon as another antiferment-inhibitor (iodin from potassium iodid) appears on the scene, the balance is disturbed and the reaction sites light up, perhaps more violently than before. The 1st antiferment-adsorber came from without (agar or luetin); the 2nd came by way of the blood (iodin). That there is no essential difference between the action of the 1st and the 2nd seems plausible, and the clinical resemblance between the reaction to iodid and the reactions to luetin and agar is rather to be expected than otherwise.

Without wishing to overrate somewhat circumstantial evidence, the author believes that a stronger case than ever before can be made for non-specificity in the strict sense, in the luetin reaction. It seems reasonably open to interpretation as, at least in part, a local adsorption phenomenon, due to anaphylatoxin liberated by uncovered proteolytic ferments from a non-specific matrix in the tissues or the serum of the syphilitic, and not in the injected spirochetes. Its legitimate function may be conceived as that of a measure of ferment activity or the lability of ferment-antiferment balance.

REACTIONS BASED ON PROTEIN AND PROTEIN-BACTERIAL EXTRACTS

The problem of accounting for intradermal reactions increases distinctly in complexity with the consideration of organ extracts and tissue suspensions, with or without a content of bacteria.

The work of a number of investigators (Dold[12] and others), has established the toxicity of organ extracts, even for homologous animals. The observations cover a wide range of tissues and various methods of preparation, including tissue juices—as from placenta—and cellular and filtered extracts in various menstrua, etc. A recent investigation by Schenk[31] ascribes the toxicity of placental juices to the presence of fibrin ferment.

[31] Ztschr. f. Immunitätsf., 1914, 22, p. 229.

While it is impossible to enter into an extended discussion of these toxic properties, the possibility of their influence on intradermal injections must be borne in mind. However, in the rapidly increasing variety of anaphylatoxin-forming substances the action of which is being ascribed to physical rather than to chemical phenomena, are included precipitated and coagulated proteins of many kinds, such as boiled antigen, boiled horse serum· (Friedberger and Castelli, quoted by Doerr⁶), boiled precipitates (Friedberger and Jerusalem cited by Doerr), in which the action of contained enzymes is ruled out, as well as such combinations as inactivated horse serum and guinea-pig serum (Friedberger[14] and Nathan[18]). The adsorptive power of placental tissue has been particularly discussed by Jobling, Eggstein and Petersen[13a] in connection with the Abderhalden reaction mentioned.

The contention is therefore fairly well grounded, that protein suspensions may act as ferment-adsorbents and give rise to anaphylatoxins by a physical mechanism of the type heretofore considered.

THE REACTIONS TO PALLIDIN AND SYPHILIN

The attempt to secure a cutaneous test for syphilis by the use of spirochete-containing organ extracts preceded, by some years, Noguchi's luetin. Liver-tissue extracts, in particular, formed the basis of such preparations as "syphilin," employed by Nicholas, Favre, Gautier and Charlet,[32,33] Fontana[34] and others, with somewhat inconstant results, approaching those later more clearly demonstrated by luetin and pallidin. "Pallidin" as employed by Klausner,[35] after Fischer,[36] is a suspension of lung tissue from pneumonia alba, rich in Spirochaeta pallida ; heated to 60 C. before use.

Of interest in this connection is Nakano's[21] observation that extracts of syphilitic liver will produce anaphylatoxin in vitro with syphilitic serum, but not with normal serum. The production of anaphylatoxin becomes more marked the older the lues from which the serum is taken. Addition of guinea-pig complement markedly increases the anaphylatoxic effect of the serum.

It is possible that the action here observed is not a simple one, and at first glance an antigen-amboceptor reaction is certainly suggested by the failure of Nakano's controls of normal-liver extract and syphilitic serum to react. It should not be forgotten, however, that spirochete-containing liver extract can behave as spirochetes would, and that the anaphylatoxin developed may be due to them. The failure of

[32] Compt. rend. Soc. de biol., 1910, 68, p. 257.
[33] Lyon médical, 1910, 114, p. 621.
[34] Dermat. Wchnschr., 1912, 54, p. 109.
[35] Arch. f. Dermat. u. Syph., 1914, 120, p. 444.
[36] Wien. klin. Wchnschr., 1913, 26, p. 49.

the extract of syphilitic liver to develop anaphylatoxin in contact with normal human serum can be accounted for by the relatively little protease (Jobling, Eggstein, and Petersen[13]) in normal human serum. However, variation in the protease and antitrypsin content of human serum has been demonstrated in several pathologic conditions, notably carcinoma, tuberculosis (Jobling), pregnancy, and pneumonia. To quote these authors, "it seems probable that in various pathological conditions proteases normally confined to the leucocytes in the human being appear in the blood, where their presence can be demonstrated by a method which removes the antiferment without injuring the ferment." Such a view applied to syphilis would provide an acceptable explanation for Nakano's observation that anaphylatoxin was generated only in contact with the pathologic (syphilitic) serum, which supposedly contains more protease than the normal. Nakano's[21] own observation that the effect was greatly heightened by the addition of a serum rich in protease (guinea-pig complement) is confirmatory of this view.

In such a reaction, then, the spirochetes conceivably act as adsorbents, liberating or uncovering the increased protease in syphilitic serum. Such protease need not be specific any more than in the case of pregnancy, carcinomatosis, tuberculosis, or pneumonia. That addition of a distinctly non-specific protease such as that of guinea-pig serum accelerates the reaction seems further to weaken the contention that the reaction is a specific one.

It seems reasonable to suppose, then, that the local reaction to syphilitic-tissue extract is to some extent the result of antiferment-adsorption by the contained spirochetes. The proteolysis which produces the anaphylatoxin is made possible by the uncovering of non-specific proteases in syphilitic blood as part of the pathologic picture of the disease. The observations of Börnstein, Nast and Nickau[19] relative to non-specific lytic effects from the serum of syphilitics will be recalled as directly in accord with this view.

On a similar basis the behavior of "pallidin" (Klausner[35]) can be accounted for, with the application of special factors in special cases. "Pallidin" as described by Klausner, who used Fischer's method of preparation, is a suspension of lung tissue and spirochetes in physiologic salt solution (0.5% phenol), heated to 60 C. Jobling, Eggstein and Petersen,[13a] in discussing the behavior of placenta in the Abderhalden reaction, mention lung tissue as second to placental tissue in efficiency, as a substrate. If this parallelism between the behavior of placental and lung tissue extends to the adsorbent properties experimentally

determined by these authors for placenta, an index to its effect in "pallidin" is obtained. The superiority claimed for "pallidin" over other spirochete-containing tissue extracts may therefore be that of a more efficient adsorbing base as well as a rich spirochetal content.

Boas and Stürüp[37] have followed up Klausner's results on pallidin, and in addition have shown that "pallidin" reactions can be produced in late syphilis by the use of extracts of chancroidal bubo taken from non-syphilitics. This observation can also be reconciled to the views here presented by recalling that such a suspension is again, a mixture of coagulated protein plus bacteria (Ducrey strepto-bacillus) and that there is reasonable ground for expecting it to have antiferment-adsorptive properties, even tho they have not as yet been demonstrated as such. The local anaphylatoxin reaction produced by such a preparation also rests on the well-supported presumption of the presence of increased amounts of non-specific proteases in the serum or tissues in late syphilis.

The reported production of luetin reactions by Boas and Ditlevsen[22] with gonococcal and colon-bacillus suspensions in late syphilis without complicating gonorrhea or gastro-intestinal symptoms, represents the non-specific anaphylatoxin reaction from bacteria minus the tissue-protein-suspension adsorbent. The author's own apparent production of a luetin reaction in a late syphilitic by an emulsion of normal skin represents at the other extreme, antiferment-adsorption and local ana-phylatoxin-formation by tissue protein minus the bacterial adsorbent.

INTRADERMAL TESTS FOR PREGNANCY

In the effort to apply the theoretical mechanism of the Abderhalden reaction as elaborated by its discoverer, to clinical conditions, several observers have undertaken intradermal tests with various extracts and fractions of placental tissue. The favorable results as to specificity reported by Engelhorn and Wintz[38] have not been corroborated by other observers, notably Esch,[39] De Jong,[40] and Falls and Bartlett.[41] De Jong, using ground placenta with ground-muscle tissue as control, found the reaction worthless in cattle. Falls and Bartlett, using whole placenta and various fractions in cutaneous, subcutaneous, and intradermal tests could find no evidence of specificity. They conclude that the pregnant woman is certainly not a sensitized woman in the usual sense.

[37] Arch. f. Dermat. u. Syph., 1914, 120, p. 730.
[38] München. med. Wchnschr., 1914, 61, p. 689.
[39] Ibid., p. 1115.
[40] Ibid., p. 1502.
[41] Chicago Path. Soc., 1915, 9, p. 249.

In the interpretation of this evidence for non-specificity Jobling, Eggstein, and Petersen's[13a] experimental studies of the marked antiferment-adsorptive capacity of placenta again apply. Taken in conjunction with the known rise in antitryptic titer in the serum of pregnant women, which implies a rise in proteases, the mechanism so often brought forward in this paper is provided for. It may be applied to this intradermal reaction precisely as in the case of the other antiferment-adsorbents heretofore considered.

EMULSIONS OF NORMAL AND PATHOLOGIC SKIN

The author's results with injections of skin emulsions may be made to harmonize logically with the foregoing. The experimental data which strengthen the case for a non-specific mechanism in the case of agar, luetin, pallidin, placentin, have not yet been developed. Clinically there is nothing suggestive of specificity about the results, even Sellei's[42] conclusions in regard to "homaesthesia" being unsubstantiated by this work. In normal skins with emulsions of normal skins, reactions as distinctive apparently as the papular luetin reaction can be produced. A provisional test with boiled emulsion makes it likely that the same reaction can be duplicated with all suggestion of ferments in the injected material ruled out. In a late syphilitic, a normal-skin emulsion produced the picture of a pronounced, tho not pustular, luetin reaction. In another psoriatic, otherwise normal, the reaction was mild, even doubtful. The anti-enzyme-adsorbent power of such suspensions would either seem to be somewhat inferior to that of suspensions containing organisms (palladin, bubo extract, etc.), or else the low protease content of normal human serum affects the reaction. The nearest approach to specificity was in the case of pityriasis lichenoides chronica, which reacted both to a psoriatic-lesion emulsion and to his own lesion emulsion on two occasions and indefinitely the third time. Even this can be easily accounted for in the theory by the marked difference in composition of the normal and pathologic emulsions. The reaction was not really specific for pityriasis lichenoides chronica, but simply for an extract of a tissue showing a marked content of scales and leukocytes, for example, typical of psoriasis as well as of pityriasis lichenoides chronica. It is of course impossible to say what difference this would make in antiferment adsorptive power. It is, however, quite as reasonable to expect a difference between normal and pathologic tissue here as between various tissues in the adsorptive phenomena of the Abderhalden reaction.

[42] Berl. klin. Wchnschr., 1910, 47, p. 1836.

MISCELLANEOUS CONSIDERATIONS

The writer has purposely limited his discussion to the reactions mentioned, in order not to obscure the issue with too great a mass of details, many of them of uncertain application. The production of local intradermal reactions by colloidal silver suspensions (electrargol) has been described by Hift,[43] and "non-protein anaphylaxis" to atoxyl and other substances has been described. Hift states that he could not secure the reaction on first injection. The status of reaction to a colloidal metal may, of course, be that of any other colloidal antiferment adsorbent, altho it may be recalled that Bordet[10] could secure no anaphylatoxin-formation with a colloid such as silicic acid. To those who interpret the behavior of adsorbents as disturbances of colloid equilibrium, however, such an explanation of the reported behavior of electrargol would be rational.

The cutaneous tuberculin reaction is also of some interest in this connection. Perkel[44] quotes Wolff-Eisner[45] as having attacked the specificity of the torpid form of this reaction on the ground that autopsy on patients presenting it showed no demonstrable evidence of tuberculosis. Killed tubercle bacilli have been shown to cause the development of bacterial anaphylatoxin in guinea-pig serum, apparently by their ferment-inhibitive capacity. Drying and grinding of tubercle bacilli does not influence their ability to produce anaphylatoxin (Doerr). The possibility that this same property is contributory to the production of seemingly non-specific and atypical von Pirquet tests, should not be overlooked.

The formation of anaphylatoxin by diphtheria toxin has been demonstrated by Friedberger and Mita[46] and its ability to inhibit antitrypsin by Jobling and Petersen.[13c] It is impossible, of course, with the experimental material at present available to generalize sweepingly in regard to the mechanism of the Schick[47] test, which depends on an intradermal reaction produced by diphtheria toxin. The reaction itself, and its value as an index of diphtheria immunity, may involve antigen-amboceptor reactions in accord with the older theory. None the less it is suggestive, that the injected toxin adsorbs antiferments in vitro. It may conceivably, in those cases in which it is not inhibited by antitoxin, uncover ferments that give rise to some extent to a proteolysis in which toxins are produced, the matrix of which is not the injected toxin, but the proteins of the patient.

The lighting-up of the original site of injection, following a 2nd injection after a supposed refractory or preparation period, in which the body produces antibodies to cope with the parenterally introduced protein, has been accepted as in line with the ordinarily conceived mechanism of active anaphylaxis. In his own experiments, the author has not thus far encountered any such reaction, regardless of the time interval between the two injections. Perkel,[44] however, working with luetin, has reported in his own case what he supposed was the lighting up of a negative site of injection after a 2nd injection 10 days after the 1st. Since torpid reactions have been known to light up at even longer intervals from the time of injection, his supposed anaphylactic reaction is open to

[43] Wien. klin. Wchnschr., 1913, 26, p. 1546.
[44] Arch. f. Dermat. u. Syph., 1915, 121, p. 7.
[45] Die Ophtalmo- und Kutan-Diagnose der Tuberculose, 1908.
[46] Ztschr. f. Immunitätsf., 1913, 17, p. 506.
[47] München. med. Wchnschr., 1913, 60, p. 2608.

the suspicion of being simply a tardive reaction. The prompter reaction in Perkel's case, with the 2nd injection, was observed by the author also in his 2nd injection of agar. The author's was made on the other arm, whereas Perkel made his within 3 cm. of the 1st. The areola of the 2nd reaction extended well around the site of reaction of the 1st. It seems possible that such close proximity led to a confusing picture. The effect of injecting a 2nd dose of antitrypsin adsorbent in the immediate field of activity of the 1st, is comparable in a local way to the effect of introducing an inhibitor of antiferment such as potassium iodid by way of the blood. The 2nd reaction is more severe, and the 1st, in which a balance had been reached, lights up when that balance is disturbed. It is not impossible that this same argument might apply in explanation of the severity, in the author's experiments, of reactions to agar which were made in regions which had already been the site of reactions to skin emulsions, etc.

SUMMARY

Recent conceptions in regard to the physical mechanism of anaphylaxis can be applied to advantage in clearing up the confusion incident to conflicting reports on the specificity of certain intradermal tests. Such reactions, including those to luetin and pallidin, that to agar observed by the author, to iodid (Sherrick), to placental substrates, and to skin emulsions, may be conceived as at least in part due to the parenteral introduction of antiferment-adsorbents, the activity of which uncovers ferments normally present in the subject. These proteases split up the proteins of the subject, with the formation of anaphylatoxins the action of which in turn produces focal necrosis and inflammation. On the possibility for a focal character of such a process, this statement by Jobling, Eggstein and Petersen[13a] is apropos: "Indeed it seems probable that the protease action can take place in what might be termed local areas of antiferment deficiency such as must occur at the point of contact of the serum and adsorbing substance." The course of the reaction is determined by the success or failure of the body cells in their effort to restore the anti-enzyme-protease balance at the site of injection of the adsorbent. The escape of locally formed toxins into the lymphatic or vascular circulation, in spite of the walling off by leukocytes characteristic of such reactions, might account for systemic symptoms. Whether the matrix of the locally formed anaphylatoxin is blood, lymph, or cellular elements of either blood or tissues, cannot be stated as yet. In general such reactions may be considered as non-specific, in opposition to the term specific as used in the antigen-amboceptor theory. They are conceivably due to the action of the patient's own enzymes on his own proteins, made possible by the inhibi-

tion of his antiferments, and not to a specific interaction between the injected substance and a specific amboceptor in the blood. The problem of why some persons react while others do not, is thus transferred, tho to a degree as yet undetermined, to an investigation of the changes which may occur in ferment balance locally in organs and tissues and in the body as a whole, within the limits of normal and pathologic processes. Experimental evidence is accumulating toward a substantial basis for such work. Future studies may well contribute a new method of approaching obscure problems in etiology and pathogenesis, especially in cutaneous diseases.

THE BACTERIA OF MILK FRESHLY DRAWN FROM NORMAL UDDERS *

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INTRODUCTION

In the early years of the science of bacteriology it was supposed that freshly drawn milk was sterile and would keep indefinitely if outside contamination could be prevented. The earliest recorded determinations of the bacterial content of such milk were made in 1891 by Schulz,[31] who reported that the first milk to be drawn contained large numbers of bacteria and that the numbers decreased as the milking progressed. But the idea prevailed for a number of years that normal milk drawn under aseptic conditions was sterile and that bacteria found in freshly drawn milk were an indication of a diseased udder. It was a mooted question until the work of Moore[23] and Ward[33] definitely proved that the normal udder may, and usually does, harbor bacteria throughout the whole extent of lactiferous ducts, including the most minute ducts, where the milk becomes contaminated as soon as secreted. This work was confirmed by De Freudenreich.[12]

Since the fact was established that milk is already contaminated before it is drawn, several investigators have studied this flora to determine the numbers and kinds of bacteria to be found there. The results of these investigators will be briefly summarized.

Gorini[14] made a study of the milk from 22 cows and found the number of bacteria varying from a sterile milk to 300,000 per cubic centimeter. The most common organisms were micrococci, which he divided into 5 types according to their growth on lactose, gelatin, and milk. In the majority of cases he found these types existing together. In the milk from 6 of the cows he found streptococci. He concludes: "The normal flora of the milk ducts of cows consists essentially of these types of cocci, among which there are always some capable of changing the milk with the same rapidity as the real lactic ferments."

De Freudenreich[13] found a varying number of bacteria with a great predominance of liquefying micrococci, some of which formed yellow, and some white colonies. He also found non-liquefying micrococci, a non-liquefying bacterium which he did not describe, and in the case of a few cows, Bacterium lactis acidi. This author noted that the bacteria which multiply in the udder

are forms which are able to withstand the bactericidal properties of the milk and tissues. He states that "these bacteria appear to be quite inoffensive."

Harrison and Savage[19] concluded that the flora of the udder is limited, and aside from that of the foremilk, which seems subject to a certain amount of change, is practically confined to a few species of white and yellow cocci, among which M. albidus and M. varians predominate. These organisms produce acid slowly in milk. M. acidi lactici was found a few times. It sometimes produces acid enough to curdle milk.

Harding and Wilson[15] studied more than 900 samples of milk taken with precautions against contamination. They found an average content of about 500 bacteria per cubic centimeter. Only 8% of the samples had a germ content of more than 1000 per cubic centimeter. Seventy-five percent of the cultures were classed as micrococci with M. albidus and M. varians occurring most frequently. Only 2 streptococcus cultures were found, and they were not typical strains. Several varieties of Bacterium were recorded under Bacterium lactis, with variety names brevis, flocculus, citronis, nonacidi, aureum II, Connii, and acidi.

Only one type of udder bacteria has been much discussed in the literature in its relation to sanitary milk. Various investigators have demonstrated the presence of streptococci in large numbers in milk coming from cows which have been affected with mastitis. Streptococci have also been found in milk coming from apparently healthy udders. In 1904, Petruschky[27] announced as a result of his investigations that the high infant mortality in summer was caused by streptococci which he found in milk in large numbers. Since then there has been considerable discussion about the significance of streptococci in milk in relation to disease. This discussion has been greatly confused by the similarity and apparently close relationship between the Streptococcus pyogenes of inflammatory processes and Streptococcus lacticus (Kruse) or Bacterium lactis acidi, as it is commonly called, the ordinary milk-souring organism which always gets into milk from external sources. In the opinion of many bacteriologists, Petruschky mistook Streptococcus lacticus for Streptococcus pyogenes.

In recent years a new interest has been directed to the presence of streptococci in milk because of several sore-throat epidemics, most notably the Chicago epidemic in 1911-12, which were caused by streptococcal infections, were shown to be milk-borne, and were traced to infected udders.

Hastings and Hoffmann[21] studied the milk of two cows throughout one or two lactation periods in each case. These cows had shown abnormally high counts in a preliminary investigation. One averaged 38,000 and the other 30,700 bacteria per cubic centimeter. The high count in both udders was due to the presence of a streptococcus, altho neither animal was known to have had garget.

Baehr[2] examined 81 samples of milk in the vicinity of Düsseldorf. Part of the samples were from milk dealers and part were from individual cows. Streptococcus pyogenes was found in only 2 of all these samples, and in the case of 1 of these 2, the milk was from a diseased udder.

Sherman and Hastings[32] obtained results very different from Baehr's. In their recent work they found streptococci in 0.01 c.c. of the mixed milk from 10 out of 12 herds and in 38.6% of the individual samples from 88 cows of 4 herds. One of these herds was well-known to the authors, and, so far as could be learned, there had been no case of udder-inflammation and no trouble had resulted from the use of this milk, which was sold largely for the feeding of children.

From all the work which has been done to show the constant presence of bacteria in freshly drawn milk it has come to be generally recognized that when the dairyman has done his best in the way of cleanliness, and when the most ideal conditions have been maintained from the time the milk is drawn until it is consumed, nevertheless it is contaminated from a source over which there can be little or no control. But with the exception of the streptococcus group very little is known about the nature of the bacteria of the udder. The literature is rich in comparative studies of strains of streptococci from different sources. The data showing the frequency of this type of bacteria in milk coming from healthy cows, however, are very meager. Certainly, in view of the fact that the number of streptococci in milk is frequently proposed as an index to its sanitary condition, this is an important consideration. Concerning the nature of the other types of bacteria in freshly drawn milk almost nothing is known. It has been assumed that they are inoffensive. But dairy bacteriology has progressed to a point where it is now desirable to have our knowledge based on something more substantial than assumption. It is a matter of especial interest to those who wish to obtain the best quality of milk for the feeding of infants or invalids, whether or not the bacteria normally present in the best of certified milks may ever be of a harmful nature, either because of their virulence or because of a possibility of their bringing about undesirable chemical changes in the milk.

The study here reported was undertaken to gain some knowledge of the frequency of the various types of bacteria normally present in freshly drawn milk, and to find out something more about the nature of those types concerning which little is known.

The Samples of Milk Studied

All the samples of milk studied were taken from 5 herds of cows which are kept in modern clean barns. Three of the dairies belong to institutions in the vicinity of Washington, D. C., and 2 supply certified milk to the city of Chicago. Altogether, samples of milk from 161 cows have been studied. From some of the cows of Dairy 1, as many as 2, 3, or 4 samples were taken from different quarters of the udder, making 192 samples of milk studied in all. The samples were obtained at milking time after part of the milk had been drawn, to avoid contamination with bacteria which might have got into the teat after the last milking. The literature shows that such are washed out during the first part of the milking. The milker would draw a stream of milk into a sterile test tube which was immediately placed in ice water, where it was kept until plating was done. The samples from 3 of the dairies were taken in the afternoon and plated the following day. The samples from Dairy 1 were plated within an hour or two after they had been taken. Those from Dairy 2 were plated about 5 hours after the milk had been drawn.

METHOD OF OBTAINING THE CULTURES

It was the intention in this study to consider only cultures which represent the types of bacteria which can multiply in the udder and become localized there, and the selection of cultures was made always with this in view. No one method of plating and isolating was used. The study of one series of samples gave suggestions for improvements in the method which were carried out on the next series of samples. It will be unnecessary to describe all the steps taken. The method used in the study of the last series of samples will be described in detail, however, for this is the most successful method found for obtaining growth of all the types of bacteria of the udder.

Among the procedures discarded as useless were the plating of samples on gelatin, and the maintenance of anaerobic conditions for incubation.

The last series of samples was plated as follows: Two sets of plates were made for each sample, each set with 1 : 10 and 1 : 100 dilutions of the milk. Into one set of plates lactose agar was poured, and they were incubated at 30 C.; into the other set of plates serum agar was poured and they were incubated at 37 C. The serum agar was made by adding 10% of cow's blood serum to the melted agar just before pouring into the plates, at about 50 C. The blood serum had been obtained with precautions against contamination. It was preserved for use in test tubes to each of which had been added a few drops of chloroform. The tubes were kept in an ice-box. When the serum was to be used it was pipetted off. The small amount of chloroform retained in the serum was driven away by the heat of the melted agar, and did not interfere with the development of colonies.

After 6 days' incubation the colonies on the plates were counted and some were picked off for inoculation. In the case of most of the samples, all the inoculations were made from the serum agar. But if the colonies were very few in number, or if for any other reason it seemed probable that a more representative set of cultures for that particular sample would be obtained by making use of the lactose agar plates, colonies were also picked off them.

For each sample of milk about 20 cultures were made, all into litmus whole milk, with precautions to make several cultures from each type of colony on the plates and to avoid colonies which appeared to be contamination. After 2 days' incubation subcultures were made on infusion-agar slopes. These were incubated for 2 days, or longer if necessary, then the cultures were examined and most of them were discarded. Every odd culture from among the 20 was discarded as possible contamination or as representing a type too few in numbers to be worthy of further consideration. A single culture of each type representing 2 or more cultures from a sample of milk was selected for further study. The selection was made by comparing all the evidence available; namely, appearance of growth in litmus whole milk and growth on agar slopes. In this way sometimes a single culture or sometimes 4 or 5 cultures were obtained from a sample of milk for further consideration. A very rough estimate of the proportion of each type present in the sample could be obtained by recording the number of cultures represented by each one saved.

By this method of obtaining representative cultures, and by discarding every one concerning which there was any suspicion that it might have been a contamination after the milk was drawn, or that it might have made its way into the teat from external sources, it was made certain that those cultures which have been studied in detail and considered in this report represent types which multiply in the udder and occur in numbers large enough to receive consideration.

Milk Samples Free from Contamination

Among the plates made from the milk of each dairy, there were some which showed no growth on the 1 : 10 dilution, the lowest dilution plated. Other plates showed only 2 or 3 colonies. When there were so few colonies, it was thought likely that they came from outside contamination. Even tho they came from the udder they did not represent a large enough number of bacteria in the milk to receive consideration; therefore, they were discarded and the samples of milk from which such plates were made were considered free from bacteria.

Altogether, 31, or 16.1%, of the 192 samples were from quarters of the udder in which bacteria were not multiplying in numbers worth considering.

Morphology of Udder Bacteria

A microscopic examination was made of the cultures obtained by the method of selection described. The slides were prepared by making a smear from the condensation water of a 24-hour growth on an agar slope, or, if the culture developed slowly, of an older growth. The slides were stained according to the Gram-Weigert method (differing from the Gram-stain in decolorizing in a mixture of 2 parts of xylol and 1 part of anilin).

Drawings of all cultures were made with the aid of a camera lucida and with a magnification of 4800 diameters on the drawing board. According to their morphology the bacteria of the udder fall under 3 groups—streptococci, micrococci, and bacilli. These groups will be considered separately.

STREPTOCOCCI

A most important matter in the study of the streptococci of milk is the differentiation between the long-chain streptococci, which may be of a virulent type, and the saprophytic Streptococcus lacticus (Kruse). It is my opinion that such a differentiation can be made by the simple method here described, altho there are undoubtedly variations which do not conform to either of the two types.

The method used in this study for identifying the streptococci was that employed by the author[10] in a previous work and used also by Sherman and Hastings[32] in their recent work. The differentiation is made roughly by the characteristic growth in litmus milk.

Long-chain streptococci usually curdle the milk, and reduction of the litmus may take place after curdling, but the color is never completely reduced. With Streptococcus lacticus cultures the reduction of

litmus precedes the curdling and is complete beneath the sharply defined pink surface layer.

The cultures differentiated by this method were examined microscopically to confirm their identity with one type or the other. Streptococcus lacticus lacks the tendency to form long chains and some or all of the cells are elongated with tapering ends.

Frequency of Streptococci in Milk from Normal Udders.—From the 192 samples of milk studied, Streptococcus lacticus (Kruse) was isolated only once. It was discarded as contamination, as were all other cultures isolated only once. From these data the conclusion may be drawn that Streptococcus lacticus does not localize and multiply in the udder. This is in close agreement with the statement made by Rogers and Dahlberg[28] that 93% of their cultures of streptococci from the udder failed to reduce litmus.

Long-chain streptococci which failed to give the reduction of litmus characteristic for Streptococcus lacticus were found in the milk from all 5 of the dairies from which samples were obtained, in numbers ranging from a very few to 264,000 per cubic centimeter. The percentages of samples which contained streptococci were for the several dairies as follows: 3.5, 10.9, 12.5, 20, 27. Altogether, streptococci were isolated from 29, or 15.1% of the 192 samples.

No detailed study was made of the streptococcus cultures, because they are the subject of study by other investigators of this division.

MICROCOCCI

Frequency of Micrococci in the Samples of Milk.—All investigators of the bacterial flora of the udder have agreed that micrococci occur there most frequently. The study here reported confirms this statement. They were found in numbers from a very few to 80,000 per cubic centimeter. The percentages of samples which contained micrococci were for the several dairies as follows: 39.3, 46.9, 47.5, 72.7, 75.7. Altogether micrococci were isolated from 113, or 58.8% of the 192 samples. The figures show merely the number of samples from which micrococci were isolated. They were undoubtedly present in more of the samples than the figures indicate, for in the case of samples containing great numbers of other forms, with comparatively few micrococci, they would sometimes fail to be isolated.

Cultural Methods for the Differentiation of Micrococci.—As a preliminary part of this study a large number of cultures of micrococci were submitted

to various tests, in order to learn which tests might be valuable for differentiation into groups. The determination of the amount of acid produced in broths containing the various carbohydrates and related substances which have been used by many investigators for differential media, was made, and of these only 6 appeared to be useful for the present study. They were dextrose, lactose, maltose, raffinose, mannite, and glycerin.

The decomposition of carbohydrates and related substances has come into common use for the classification of bacterial cultures, but with the exception of the reduction of nitrates, the formation of indol, and the liquefaction of gelatin, the action of bacteria on nitrogen-containing substances has been little used for purposes of classification. It seemed, however, that the ability to utilize simple nitrogenous compounds in synthetic media should give information concerning another phase of the physiologic activities of the cultures. Accordingly, inoculations were made into media containing as the only source of nitrogen simple compounds such as the amino-acids and the nitrate and ammonia compounds. Three of these were chosen as useful in this study of the micrococci. They were reduction of nitrates to nitrites and decomposition of asparagin and urea. Gelatin-liquefaction, pigment-formation, hemolysis, and action on litmus milk were also used as differential tests.

Pigment-Formation.—In order to determine the pigment-formation under uniform conditions, strokes were made on infusion-agar slopes and incubated for 2 weeks at 20 C. The growth was removed and spread thickly on a piece of white drawing paper. It was then compared immediately while moist with the color plates in Ridgway's "Color Standards and Nomenclature." This method for determining pigment-formation accurately was used in an earlier work[20] on bacteria of the colon type. The explanation of the symbols used to designate the color standards (see Table 1) is quoted from that paper:

"Colors are designated by letters as 'oy,' which indicates a color made up of 65 percent orange and 35 percent yellow; 'yoy,' 47 percent orange and 53 percent yellow; or 'oyy,' 25 percent orange and 75 percent yellow.

"Variations from the pure colors are obtained by mixtures of neutral gray. For instance, 17 oy is a pure color; 17′oy contains 32 percent neutral gray; 17″oy, 58 percent neutral gray.

"Finally, tints, designated by lower-case letters, are obtained by mixing varying proportions of white with the pure colors, and shades by mixing black. The tint nearest the pure color is designated by the letter b; that nearest the pure white by f; the shade nearest the pure color is designated by the letter i."

Gelatin-Liquefaction.—To determine the liquefaction of gelatin a drop of broth culture was spread on the surface of a tube of infusion gelatin, the surface of the gelatin was marked on strips of paper on opposite sides of the tube, and the cotton plug was paraffined to prevent evaporation. After 30 days' incubation at 20 C. the tubes were examined. If liquefaction had occurred it was measured and expressed in millimeters of liquefaction.

Hemolysis.—Blood-agar plates were made by adding 10% of defibrinated cow's blood to infusion agar at about 50 C. and pipetting 5 c.c. of this into each plate. When the agar had solidified streaks of the culture were made on it and the plate placed in the incubator at 37 C. Ordinarily those cultures showing hemolysis within 24 hours are designated positive and those that fail to show hemolysis in 24 hours are designated negative. But in this study it was desirable to show relationships between such positive- and negative-reacting cultures. Therefore the plates were incubated for 4 days, and slight hemolytic

action was recorded. The following scheme for recording hemolysis serves to show the relationship between active hemolytic cultures and those in which this power appears to be lacking.

Very active hemolysis in 24 hours.................. $+++++$
Moderate hemolysis in 24 hours................... $+++$
Hemolysis in 2 days.............................. $++$
Slight hemolysis in 3 or 4 days................... $+$
No hemolysis .... $—$

Nitrate-Reduction.—Nitrate-reduction was determined in a peptone-free solution. Ordinarily the reduction of nitrate is determined in a peptone broth. But on the general principle that the simplest possible medium is the best for bacteriologic determinations, the peptone-free solution was chosen as more reliable after a trial of both kinds of media. The results reported in this paper are, therefore, not comparable with other investigators' data on nitrate-

TABLE 1
CONSTANCY OF CULTURAL CHARACTERISTICS

Culture	Date Tested		Action in Milk	Dextrose	Lactose	Maltose
ab	Oct.,	1913	Curdled in 14 days................	4.60	3.75	3.45
	April,	1915	Curdled in 11 days................	4.60	3.65	3.90
aj	Oct.,	1913	Curdled in 4 days.................	3.75	1.50	3.30
	April,	1915	Curdled in 6 days.................	3.20	2.25	4.30
as	Oct.,	1913	Acid, not curdled, in 14 days......	4.00	3.30	....
	April,	1915	Acid, not curdled, in 14 days......	2.30	3.70	4.10
bh	Oct.,	1913	Curdled in 4 days.................	4.45	4.25	4.35
	April,	1915	Curdled in 12 days................	4.25	3.15	3.80
bu	Jan.,	1914	Curdled in 14 days................	3.90	3.95	2.55
	April,	1915	Curdled in 14 days................	4.50	4.40	4.10
ci	Jan.,	1914	Curdled in 5 days.................	2.90	2.15	2.55
	April,	1915	Curdled in 6 days.................	3.55	2.70	4.25
dx	March,	1914	Slightly acid in 14 days...........	3.90	3.90	3.30
	April,	1915	Slightly acid in 14 days...........	4.65	4.70	4.30
fj	April,	1914	Slightly acid in 14 days...........	4.05	3.80	3.25
	April,	1915	No change in 14 days.............	4.35	4.15	4.20

reduction by micrococci. The medium was made up as follows: 1 liter water, 1 gm. magnesium sulfate, 2 gm. dibasic potassium phosphate, 1 gm. dextrose, and 3 gm. potassium nitrate.

The reagent for detecting nitrates was made up as follows[22]: (1) A solution of naphthalamin, 0.1 gm., in 20 gm. distilled water, was boiled, cooled, filtered, and 156 c.c. of dilute (1:16) acetic acid added. (2) Sulfanilic acid, 0.5 gm., was added to 150 c.c. dilute acetic acid. The two solutions were mixed in equal proportions before use.

The cultures were incubated for 7 days and then tested by adding a few drops of the reagent to 1 c.c. of the culture in a 10 mm. chemical test tube.

Decomposition of Asparagin.—Asparagin medium was made with 1 gm. magnesium sulfate, 2 gm. dibasic potassium phosphate, and 5 gm. asparagin in

1 liter of water. The cultures were incubated for 7 days at 30 C. and then tested for ammonia with Nessler's reagent.

Decomposition of Urea.—The urea was sterilized in a solid condition by heating for half an hour in a toluene oven at 105 C. This precaution is necessary, for urea in solution is broken down to ammonia during the process of sterilization. Tubes of salt solution (1 gm. magnesium sulfate and 2 gm. potassium dibasic phosphate in 1 liter of water) were sterilized and then a small piece of the sterile urea was placed in each tube. After 7 days' incubation at 30 C. the cultures were tested for ammonia with Nessler's reagent.

Fermentation Tests.—The media for the fermentation tests were made as follows: Beef extract, 4 gm.; peptone, 10 gm.; dibasic potassium phosphate, 5 gm.; test substance, 10 gm.; and water, 1000 gm.

After 7 days' incubation at 30 C., 5 c.c. of the culture were titrated in the cold with phenolphthalein as an indicator against 0.05 normal sodium hydroxid.

TABLE   1—*Continued*
CONSTANCY OF CULTURAL CHARACTERISTICS

Raffinose	Mannite	Glycerin	Lique-faction of Gelatin	Decom-position of As-paragin	Decom-position of Urea	Chromogenesis
.30	.75	.10	....	—	+	19 YOY, light cadmium
.05	1.00	.30	....	—	—	19 YOY, light cadmium
.85	1.25	1.40	....	--	+	15 YOb, capucine yellow
.90	.90	.70	....	--	—	15 YOb, capucine yellow
.15	.05	.40	....	—	—	21″ OYY f, ivory yellow
.05	.30	.40	....	--	—	21″ OYY f, ivory yellow
.20	— .15	1.35	....	—	+	21″ OYY f, ivory yellow
— .05	.05	1.25	....	—	—	19′ YOY f, cream color
.00	.10	.30	....	—	+	19′ YOY f, cream color
.20	.20	.25	....	--	—	19′ YOY f, cream color
.90	1.45	.80	17 mm.	—	+	17 OY f, pale orange yellow
1.00	1.10	.75	16 mm.	—	—	19′ YOY f, cream color
.40	2.25	.65	20 mm.	—	+	17 OY b, deep chrome
.35	1.60	.65	15 mm.	—	—	15 YO b, capucine yellow
.15	1.35	.25	....	—	+	17 OY, cadmium yellow
.20	.95	.25	....	—	—	19 YOY, light cadmium

The acidity was recorded in cubic centimeters of n/20 NaOH with the acidity of a control tube subtracted.

Constancy of the Characteristics of Individual Cultures.—A question always of interest when cultures are classified according to certain characteristics is whether the characteristics are constant. A number of the cultures of micrococci were submitted to the tests a second time after being maintained a year or more on agar slopes. The results of the 1st and 2nd tests are given in Table 1 for comparison. Hemolysis and nitrate-reduction are omitted because these tests were not made until the cultures had been maintained on agar slopes for about a year. The gelatin-liquefaction of most of the cultures is omitted for the same reason.

As may be observed by a study of the table, the cultural characteristics, with the exception of urea-decomposition, were remarkably constant. The

cultures seem to have lost the ability to decompose urea, for most of them reacted positively in the 1st test and all reacted negatively in the 2nd test. It is possible that some error may have brought about this discrepancy and not too much stress is to be laid on it until more is known about this character test.

The tests on all the 6 fermentable substances were absolutely consistent in the two determinations. Most surprising, however, is the agreement of the 1st and 2nd determinations of pigment-formation. The pigment was matched to the same color plate in both tests in the case of 4 of the 8 cultures. For the other 4 cultures the differences in pigment between the 2 tests were very slight, as any one familiar with the color chart may observe from the table. For example, Culture d.r was matched to the deep chrome chart in the 1st test, and to the capucine yellow chart in the 2nd test. These two charts are of the same tint, as indicated by the letter "b." They differ only in the presence of a very little more yellow in the deep chrome chart. The difference is so slight that an untrained eye might easily confuse the two colors.

Chromogenesis has always been regarded as an extremely variable character. But the data in Table 1 show that this is a stable character in the micrococci from the udder when determined with accuracy after growth under standard conditions.

Classification of the Micrococci from the Udder.—Micrococci coming from the udder have been called by many names. Micrococcus lactis with the variety names acidi, albidus, and varians is found most frequently. Gorini[15, 16] includes them in his group of Micrococcus casei acido-proteolyticus. He[17] ascribes a certain connection between the vigorous development of this acido-rennet flora in the udder and certain inflammatory conditions. The literature contains no record of Staphylococcus pyogenes having been found in milk freshly drawn from normal udders, altho in their description of M. lactis varians, Conn, Esten, and Stocking[5] state that the characters of this organism "agree essentially with those of Staphylococcus pyogenes aureus." They also state that they are "inclined to think that our type is the common Staph. pyogenes aureus."

A careful study of the micrococci from the udder showed that the great majority of them belonged to one type. The 185 cultures belonging to this type were arranged into groups according to their fermentation of the carbohydrates and alcohols used as test substances (Table 14). A study of the 8 groups thus classified showed that the other characteristics, action on litmus milk, hemolysis, liquefaction of gelatin, nitrate-reduction, decomposition of urea, and chromogenesis, varied within the 8 groups without any correlation. This method of classification, which has been found useful for the differentiation of other types of bacteria, has little or no value in the differentiation of this type of udder bacteria into varieties with the exception of the mannite test, to be discussed.

Three characteristics were evidently correlated ; namely, hemolysis, pigment-formation, and mannite-fermentation. Attempts were therefore made to divide the cultures into natural groups. In differentiating the groups, hemolytic action which was evident in 24 hours was called positive and hemolytic action evident later than this was called negative. The formation of pure colors and the deepest tints indicated by "b" and "d" was considered positive pigmentation and the formation of paler tints indicated by "f" or "slight pigment" was considered negative. The production of 0.5 c.c. or more of 0.05 normal acid per 5 c.c. of culture medium was considered positive fermentation.

A tentative division of the cultures into 2 groups was made first on the basis of hemolytic action, then on the basis of mannite-fermentation, and finally on the basis of pigment-formation. All these methods of division gave the same 2 general groups, the one with positive hemolytic action, positive fermentation of mannite and positive pigment-formation, and the other group with these characteristics negative. A consideration of the groups according to the 3 methods of division showed that the type characteristics were most distinct when the division was made on the basis of mannite-fermentation. This classification was therefore adopted, and the data are presented in Table 2 and graphically in Figure 1. The group showing positive mannite-fermentation, positive hemolytic action, and positive pigment-formation was recognized as identical with the Staphylococcus aureus in medical literature.

Here it will be necessary to make a short digression to discuss the pyogenic staphylococci.

These are the organisms which cause furuncles, abscesses, suppuration, and septicemia, either alone or together with Streptococcus pyogenes. A deep yellow-orange pigmentation has long been associated with the most virulent type and accordingly this has been named Staphylococcus aureus. Andrewes and Gordon[1] studied the biologic characters of the staphylococci pathogenic for man, and found mannite-fermentation to be absolutely constant for all their cultures from pathogenic sources, whereas their cultures from other sources failed in this respect. They laid great stress, therefore, on the mannite test as of high differential value.

Staphylococcus albus is known as a less virulent variety which fails to form the yellow pigment.

In a recent work Dumas[8] studied 18 cultures from pathogenic sources, and found that all fermented dextrose, lactose, saccharose, maltose, and mannite but that only 2 fermented glycerin. The hemolytic power varied greatly. Nicolle and Césari,[23] in a consideration of the whole group of staphylococci from the skin and mucous membrane and from various suppurative processes, conclude that the non-chromogenic staphylococci are usually non-virulent.

The statements of the authors quoted may be taken as a general summary of the characteristics of the pyogenic staphylococci found in the literature.

In regard to the relationship between hemolytic action and virulence of staphylococci opinions differ from that of Flügge,[11] who states that strong hemolytic action and positive agglutination are a criterion for pathogenicity, to that of Bruck and Hidaka,[4] who report that of 17 cultures isolated from cases of eczema only 35% formed hemolysin. The consensus of opinion, however, is that hemolysis may be taken as an indication of virulence, altho some virulent strains fail to hemolyze blood corpuscles and some non-virulent strains may be active in this respect. The virulence tests reported in the succeeding pages show that strong hemolytic action may be taken as an indication of virulence.

A consideration of the two groups of staphylococci as presented in Table 2 and graphically in Figure 1 shows that the group with positive fermentation of mannite, hemolysis, and pigment-formation agrees with Staphylococcus aureus in its generally accepted characteristics.

It will be observed that most of the characteristics of the staphylococci as shown in Table 2 and Figure 1, do not serve in any way to differentiate the 2 varieties. Decomposition of asparagin was negative for all the cultures of the group. Dextrose- and lactose-fermentation were positive for all the cultures. Glycerin-fermentation was sometimes positive and sometimes

TABLE 2

COMPARISON OF THE TWO VARIETIES OF STAPHYLOCOCCI FROM THE UDDER ON THE BASIS OF MANNITE-FERMENTATION

	Mannite	Hemolysis		Dextrose		Lactose		Maltose	
		+	—	+	—	+	—	+	—
Number of cultures	+	49	46	95	0	95	0	95	0
Percentage	..	51.6	48.4	100	0	100	0	100	0
Number of cultures	—	14	76	90	0	90	0	85	5
Percentage	..	15.5	74.5	100	0	100	0	94.4	5.6

negative, but this reaction was not correlated with the other characteristics of the 2 varieties. The same may be said of the urea-fermentation. The action in litmus milk also appeared to have little significance.

The comparatively few cultures which failed to liquefy gelatin had this characteristic apparently uncorrelated with any other deviation from the type. And those cultures which gave a positive reaction liquefied the gelatin in varying degrees from a decided action to a very slight action. It is only a short step from slight liquefaction to no liquefaction. Therefore no especial significance is attached to the non-liquefaction of gelatin.

It must not be inferred that because the cultural studies discussed here have had no value in differentiating the staphylococci into varieties they have been useless. It is only by an investigation of the many possible physiologic activities of a large number of cultures that the general characteristics of a type may be learned.

It will be observed that of the cultures which failed to ferment mannite, almost all failed to hemolyze blood corpuscles and failed to form pigment in the majority of cases. The few cultures which failed to ferment maltose all belonged to this group.

Those cultures which fermented mannite also formed a pigment in most cases and about half of this group hemolyzed blood corpuscles. Practically all those cultures which fermented raffinose and those cultures which reduced nitrates belonged to this group.

The close relationship of the two varieties is shown by the many characteristics which they possess in common. The interrelation of the variety characteristics shows that no hard and fast lines can be drawn to differentiate them. For example, Culture *qb* (see Table 3) failed to ferment mannite and formed only a slight pigment, yet it hemolyzed blood corpuscles very actively. Many such interrelations between the two varieties were evident. Moreover, most of the cultures for which the hemolytic action was considered negative in differentiating the two varieties were not absolutely without hemolytic action, but possessed this function to a slight degree.

If earlier classifications of the staphylococci are accepted, the negatively reacting variety from the udder would be termed Staph. epidermidis [albus]. According to Andrewes and Gordon[2] Staph. epidermidis [albus] is differentiated from Staph. albus by its failure to ferment mannite. The Winslows[34] accepted this classification, but designated the type which fails to ferment mannite by the name Albococcus epidermidis. According to these same authorities, Staph. albus, or Albococcus pyogenes as the Winslows call it, is a white nonvirulent variety which ferments mannite.

TABLE   2—*Continued*

COMPARISON OF THE TWO VARIETIES OF STAPHYLOCOCCI FROM THE UDDER ON THE BASIS OF MANNITE-FERMENTATION

Raffinose		Mannite		Glycerin		Gelatin		Nitrate		Urea		Asparagin		Pigment	
+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
32	63	95	0	45	50	85	10	15	80	71	24	0	95	77	18
33.7	66.3	100	0	47.4	52.6	89.5	10.5	15.8	84.2	74.7	25.3	0	100	81.0	19.0
2	88	0	90	57	33	79	11	3	87	60	30	0	90	35	55
2.2	97.8	0	100	63.3	36.7	87.7	12.3	3.3	96.7	66.7	33.3	0	100	38.9	61.1

After a careful consideration of all the data in regard to the staphylococci from the udder, it seemed most reasonable not to follow the generally accepted classification in which the failure to ferment mannite would place a culture in the Staph. epidermidis species, but to designate the negatively reacting variety as Staph. albus.

Because of the undoubted agreement of the micrococci of the udder with the staphylococci, the author suggests that the names Micrococcus albidus, Micrococcus varians, Micrococcus acidi, and all other names which have been applied to those micrococci of the udder which conform to this type, be dropped, since their use only leads to confusion, whereas simplicity in nomenclature is greatly needed.

Virulence of Staphylococci From the Udder.—According to the cultural tests, 95 of the 185 cultures of the staphylococci which were studied belonged to the aureus variety. It was a matter of importance, therefore, to find out whether these strains might be virulent. A number of rabbit inoculations were made to determine the virulence of the

cultures. (The writer wishes to express her thanks to Dr. George M. Potter, of the pathological division of this bureau, for making the animal inoculations and the postmortem examinations.) The cultures had been grown on agar slopes for periods varying from 2 to 16 months before the inoculations were made.

A part of the inoculations were made intravenously. According to Dreyer,[7] inoculation into the knee joint is a more delicate test for virulence. A part of the inoculations were made by this method. The results of the rabbit inoculations are given in Table 3. The mannite-fermentation, hemolysis, and chromogenesis of the cultures, characteristics of interest in respect to virulence, are tabulated there also. The data are arranged in the order of the virulence of the cultures.
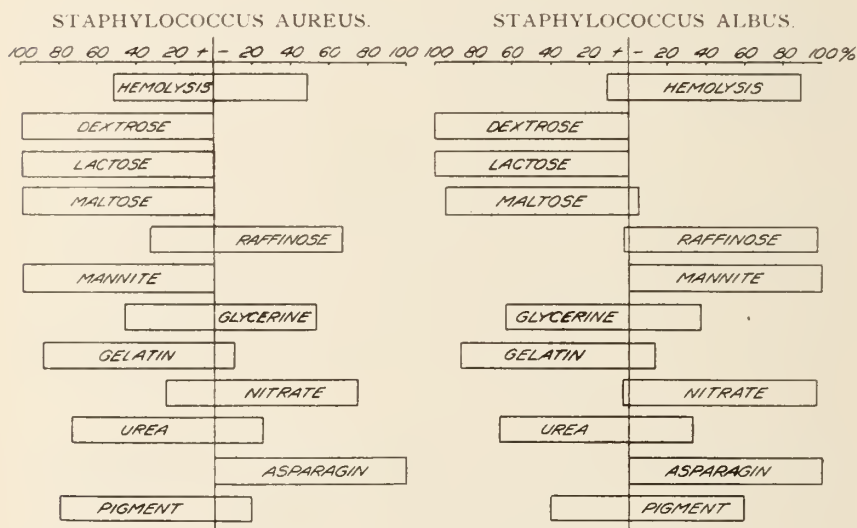
Fig. 1. Diagram showing the characters of Staphylococcus aureus and Staphylococcus albus cultures from normal udders.

Two of the cultures, *nq* and *ni*, proved to be exceedingly virulent. The inoculations were made in the afternoon, and death occurred during the following night. Six of the cultures, *nh, po, dx, dq, ib,* and *cb* produced severe local swellings, and the 2 animals which were not killed within a few days after inoculation died on the 11th and 14th days, respectively. Two other cultures, *mk* and *pc*, produced only slight swellings. The remaining 7 of the total 17 cultures inoculated showed no virulence.

TABLE 3
Virulence of Staphylococci From the Udder

Culture	Mannite Fermentation	Hemolysis	Chromogenesis	Result of Intra-articular Inoculation	Result of Intravenous Inoculation	Recovery of Organism
nq	1.85	++++	17 OY b, deep chrome	....	(1) Died within 18 hours (2) Died in 15 hours	No postmortem Cultures obtained from thoracic fluid and spleen
nɩ	1.60	++++	17 OY, cadmium yellow	Died within 18 hours	Died within 18 hours	No postmortem Cultures obtained from heart's blood and spleen
nh	1.85	++++	17 OY b, deep chrome	Joint badly swollen on 2nd day. On 6th day rabbit killed. Joint and surrounding muscle purulent	....	Culture obtained from the joint
po	2.05	++++	17'OY b, antimony yellow	No swelling on 2nd day. On 6th day killed. Joint and surrounding muscle purulent	....	Culture obtained from the joint
dx	2.25	++++	17 OY b, deep chrome	Joint badly swollen on 3rd day. Rabbit died on 14th day	Very sick on 3rd day. Slowly recovered. Killed on 19th day	Failed to obtain culture from heart's blood and spleen No growth from inoculations from heart's blood and spleen
dq	1.30	++++	17 OY, cadmium yellow	Joint swollen on 3rd day. Rabbit died on 14th day	....	No postmortem
ib	3.10	++++	19″ YOY, honey yellow	.... Joint badly swollen on 2nd day. Rabbit killed 6th day. Joint and surrounding muscle purulent	No effect	Culture obtained from the joint
eb	3.20	+++	17 OY, cadmium yellow	Joint badly swollen on 3rd day. Rabbit killed on 9th day	....	Culture obtained from the joint
mk	.05	+++	23′ Y, strontian yellow	Joint slightly swollen and fever on 3rd day. A very little swollen on 8th day. Later, rabbit recovered		
pe	.20	++++	15 YO, orange....	.... Joint slightly swollen on 3rd day. No swelling on 6th day. Rabbit killed on 9th day	No effect	No growth from inoculations from heart's blood, joint, and spleen

TABLE 3—*Continued*
VIRULENCE OF STAPHYLOCOCCI FROM THE UDDER

Cul-ture	Mannite Fermentation	Hemolysis	Chromogenesis	Result of Intra-articular Inoculation	Result of Intravenous Inoculation	Recovery of Organism
df	2.10	+ +	17 OY b, deep chrome	No effect		
kz	1.15	+ + +	17 OY, cadmium yellow	No effect		
my	.10	+ +	23″ Yf, marguerite yellow	....	No effect	
qb	.00	+ + + +	19′ YOY f, cream color	No effect...........	No effect	
qy	1.35	+ + + +	15 YO d, orange buff	No effect...........	No effect	
rz	1.75	+ + +	17′OY b, antimony yellow	No effect...........	No effect	
sa	.20	+	13 OYO, cadmium orange	....	No effect	

In selecting the cultures for the virulence tests, a part were chosen because their active hemolysis and positive mannite-fermentation and pigment-formation indicated that they would be virulent. A number of the cultures were chosen for the purpose of determining the pathogenic properties when there was a variation in one or more of these characteristics.

It is an interesting fact that all 8 of the cultures which possessed sufficient virulence to cause serious results were mannite-fermenters which hemolyzed actively and formed a deep-colored pigment. But the non-virulent cultures and those showing slight virulence failed to give a positive reaction in one or more of the 3 positive characteristics of the aureus variety.

Frequency and Distribution of Virulent Strains of Staphylococci —It is impossible to estimate from the results with the 17 cultures which were inoculated what proportion of the 185 cultures possessed virulence. Since all the cultures which showed an unusually active hemolysis were inoculated into rabbits, it is probable that all the most virulent cultures were tested for their pathogenic properties. The two exceedingly virulent cultures, *nq* and *ni*, were both isolated from samples of milk from Dairy 4. Culture *nh*, with somewhat less virulence, was also from Dairy 4. The virulent culture *po* was from Dairy 5 and the other four virulent ones were from Dairy 1. The cultural tests indicated that none of the strains from Dairies 2 and 3 was virulent. Cultures *kz* from Dairy 2 and *mk* from Dairy 3 were chosen for inoculation as the most probably virulent of all the strains

from those two dairies. Culture *kz* proved to be non-virulent and Culture *mk* possessed only a slight virulence.

An estimation of the number of organisms of the virulent strains present in the milk from which they were isolated is given in Table 4. It may be noted that they vary from a very few to 80,000 per cubic centimeter.

TABLE 4

FREQUENCY OF VIRULENT STRAINS OF STAPHYLOCOCCUS AUREUS

Culture	Dairy	Estimated Number per c.c. of Milk
nq	4	50
ni	4	1600
nh	4	80,000
po	5	4000
dx	1	32,000
dq	1	270
ib	1	11,600
eb	1	30

Coccus Cultures not Included with the Staphylococci.—Not all the micrococci obtained from the udder were classed with the staphylococci. First among these to be discussed is a small group which was characterized by a negative reaction to most of the tests. Four of the 8 cultures gave too scant growth for the determination of pigment. These 4 cultures died after being maintained a few months on agar slopes. The other 4 cultures were re-inoculated in the test substances after a year's cultivation on agar slopes. At the time of the 2nd tests the cultures gave reactions similar to those of the staphylococci. It appears that the cultures had acquired the ability to ferment the test substances. But there is a possibility that some peculiar conditions which escaped observation might have prevented positive reactions in the 1st series of tests. Because of their peculiarity in the 1st tests these cultures have been omitted from the staphylococci to simplify the discussion.

Three other groups of micrococci from the udder gave reactions which were considered to differ from those of staphylococci sufficiently to separate them into distinct groups. One of these is presented in Table 5. The lack of any hemolytic action whatever, and the decomposition of asparagin, distinguish this group from the staphylococci. The group can not be identified with any described species, because its classification was made largely by means of its ability to decompose asparagin, a character heretofore not used in

classification. No name will be suggested for the group because too few cultures of it have been studied. Cultures of this group were isolated from only 4 samples of milk. These samples were all from Dairy 5.

TABLE 5
ASPARAGIN-DECOMPOSING, NON-HEMOLYTIC MICROCOCCI

Culture	Dairy	Cow	Action on Milk	Dextrose	Lactose	Maltose	Raffinose
sb	5	59	Acid, not curdled, in 14 days	3.35	3.05	2.05	—.05
sf	5	68	Acid, not curdled, in 14 days	3.50	3.30	1.65	—.10
sm	5	75	Acid, not curdled, in 14 days	3.25	3.00	2.05	.00
rr	5	56	Acid, not curdled, in 14 days	3.10	3.20	2.20	—.15

Another group of micrococci from the udder is presented in Table 6. The group is characterized by the non-liquefaction of gelatin and the ability to decompose both urea and asparagin. Dextrose is fermented but the ability to attack the other test substances is characteristically lacking. This group has been designated M. luteus, the name adopted by the Winslows[34] for cocci of this description. Three of the cultures do not conform with their description of this species in the positive

TABLE 6
MICROCOCCUS LUTEUS

Culture	Dairy	Cow	Action on Milk	Dextrose	Lactose	Maltose	Raffinose
af	1	1	No change in 14 days	4.25	.05	1.30	.25
ag	1	1	Acid, not curdled, in 14 days	6.55	2.35	.25	.10
ah	1	1	Acid, not curdled, in 14 days	4.45	2.90	.25	.10
db	1	27	No change in 14 days	1.70	— .05	.30	.05
fu	1	21	No change in 14 days	1.00	.95	.60	.00
py	5	30	No change in 14 days	1.20	—.20	.60	—.30

reaction in lactose broth, and 3 of the cultures disagree with their description in the reduction of nitrates. But all 6 of the cultures in Table 6 appear to belong to a related group, and the discrepancies mentioned are certainly not sufficient to give the group another name.

The last group of micrococci to be considered is presented in Table 7. This group is distinguished from all other udder organisms by the rapid and complete peptonization of the milk. Gelatin is liquefied, and dextrose is fermented. Maltose, mannite, and glycerin are usually

TABLE   5—*Continued*
ASPARAGIN-DECOMPOSING, NON-HEMOLYTIC MICROCOCCI

Mannite	Glycerin	Hemol-ysis	Liquefaction of Gelatin	Reduction of Nitrate	Decomposition of Asparagin	Decomposition of Urea	Chromogenesis
1.75	1.65	—	20 mm.	—	+	—	19″ YOY f, cartridge buff
1.35	.70	—	25 mm.	—	+	—	19″ YOY f, cartridge buff
2.10	2.20	—	25 mm.	+	+	—	19″ YOY f, cartridge buff
1.95	.70	—	18 mm.	—	+	—	19″ YOY f, cartridge buff

fermented. Hemolysis is very slight or negative. In respect to the nitrogenous test substances there is a considerable difference between the Cultures *ao, bf,* and *bc,* which reduce nitrates and decompose asparagin but not urea, and Cultures *oc* and *ov;* they do not reduce nitrate and they decompose urea but not asparagin. But because of the small number of cultures obtained it did not seem advisable to separate the group. This group could not be identified with any pre-

TABLE   6—*Continued*
MICROCOCCUS LUTEUS

Mannite	Glycerin	Hemol-ysis	Liquefaction of Gelatin	Reduction of Nitrate	Decomposition of Asparagin	Decomposition of Urea	Chromogenesis
.30	.35	+	—	—	+	+	Slight pigment
—.15	—.05	+++	—	—	+	+	19 YOY f, maize yellow
—.45	.10	+++	—	—	+	+	19′ YOY b, mustard yellow
.85	.10	+	—	+	+	+	21 OYY f, Baryta yellow
—.05	—.15	—	—	+	+	+	21 OYY f, Baryta yellow
.05	.00	+++	—	+	+	+	17′ OY, yellow ochre

vious description of micrococci, altho it seems probable that this group, as well as the staphylococci has been included by Gorini[15, 16] in his Micrococcus casei acido-proteolyticus. The name Micrococcus caseo-lyticus is here suggested for this type characterized by the rapid

and complete peptonization of milk and by other characteristics as shown in Table 7. This type of organism is apparently widely distributed, for cultures were isolated from samples of milk taken at 4 of the 5 dairies.

TABLE 7
MICROCOCCUS CASEOLYTICUS

Culture	Dairy	Cow	Action on Milk	Dextrose	Lactose	Maltose	Raffinose
ao	1	2	Peptonized in 5 days	3.85	.10	3.10	.40
be	1	2	Peptonized in 14 days	3.75	— .10	1.85	.30
bf	1	2	Peptonized in 12 days	4.00	.15	1.40	.40
qs	5	39	Peptonized in 14 days	1.85	.05	1.50	—.05
rc	5	46	Peptonized in -4 days	1.80	— .05	1.50	—.15
me	3	327	Peptonized in 4 days	5.45	.80	6.30	.50
oe	4	74	Peptonized in 6 days	3.00	3.10	.45	.15
ov	5	11	Peptonized in 5 days	1.20	3.40	1.65	.15

Localization of Peculiar Species and Strains.—An interesting fact observed in a study of the tables is the localization of peculiar species and strains. There are many examples of this, but only a few of the most striking will be mentioned. The isolation from 4 samples of one dairy of the non-hemolytic asparagin-decomposing micrococci (see Table 5) has already been noted. Among the staphylococci, the cultures from the milk of Dairy 2 which hemolyzed actively, all failed to liquefy gelatin. From the milk of Dairies 1 and 5 a number of cultures were isolated which had the peculiarity of fermenting all 6 of the test substances. From these same milk samples several cultures were isolated which were distinguished from all the others by the fermentation of only 2 of the test substances, dextrose and lactose. Also the cultures of the M. luteus type (see Table 6) were all from the milk of these two dairies. Interesting in this connection is the fact that Dairies 1 and 5 are only a few miles apart.

Localization of Various Micro-organisms in Certain Dairies.—Not only do peculiar strains of the organisms commonly present in udders become localized, but also organisms of an entirely different nature sometimes become established in the udders of many cows of a dairy. Moore and Ward[24] found a gas-producing bacterium in the udders of many cows of one herd. A streptothrix was found in 6 of the 28 sam-

ples of milk from Dairy 3. In one sample there were 5000 of these organisms per cubic centimeter. No study was made of the streptothrix. A gram-negative bacterium which made on agar a luxurious growth

TABLE 7—*Continued*
MICROCOCCUS CASEOLYTICUS

Mannite	Glycerin	Hemolysis	Liquefaction of Gelatin	Reduction of Nitrate	Decomposition of Asparagin	Decomposition of Urea	Chromogenesis
.95	.40	Slight	18 mm.	+	+	—	21″ OYY f, ivory yellow
1.45	.35	Slight	20 mm.	+	+	—	21″ OYY f, ivory yellow
.80	.10	Slight	20 mm.	+	+	—	21′ OYY f, massicot yellow
.85	.70	—	25 mm.	—	—	—	19″ YOY f, cartridge buff
.90	.65	—	22 mm.	—	—	—	19″ YOY f, cartridge buff
4.25	1.25	—	60 mm.	—	—	—	23″ Yellow f, marguerite yellow
.40	.50	Slight	40 mm.	—	—	+	15′ YO, ochraceous orange
.25	.60	Slight	53 mm.	—	--	+	17′ OY, yellow ochre

of a peculiar yellow shade was isolated from 4 of the 40 samples from Dairy 2. These cultures did not stand the artificial cultivation, all dying in the 3rd transfer before a detailed study of them had been made. But enough is known of them to warrant the statement that they were unlike any other organism isolated from the other samples of milk.

BACILLI COMMON IN NORMAL MILK

Besides the two groups of bacteria already discussed, which are commonly present in large numbers in milk freshly drawn from normal udders, namely, the streptococci and the staphylococci, a third group of organisms was found in this study to be common in the milk from all 5 of the herds from which samples were examined. These were bacilli of the Bacillus abortus type. No description of these organisms has been found in dairy literature. But Schroeder and Cotton[3] reported B. abortus to be common in milk. They determined its presence by inoculation into guinea-pigs. Whenever B. abortus was present in the milk, there was a slow development of lesions which were characteristic for this organism.

A preliminary report[9] of the demonstration of these organisms by cultural methods has already been made. In this report, only one variety of bacillus from the udder was mentioned. It was described

under the name of Bacillus abortus, for it resembled the published descriptions of the bacillus of contagious abortion.

Further studies of the bacilli from the udder have been made, and it is now recognized that they do not all belong to a single variety but that there is a related group of bacilli common in milk from normal udders which will be called the Bacillus abortus type.

A special method for plating the milk is necessary to develop colonies of the Bacillus abortus type. A milk containing hundreds of thousands of these organisms might pass the ordinary routine bacteriologic analysis of milk without revealing a single colony. This explains why they never have been mentioned in dairy literature.

If milk samples are plated on serum agar, however, and incubated for several days, as directed in this paper under "Methods of Obtaining Cultures," the B. abortus type develops readily. When 2 sets of plates are made from samples of freshly-drawn milk, one on lactose agar and the other on serum agar, a significantly larger number of colonies on the serum-agar plates is rather certain evidence of the presence of bacilli of B. abortus type.

TABLE   8

FREQUENCY OF THE BACILLUS ABORTUS GROUP IN FRESHLY DRAWN MILK

Dairy	Number of Samples Examined	Number of Samples from Which B. Abortus was Isolated	Percentage of Samples from Which B. Abortus was Isolated	Lowest and Highest Numbers of B. Abortus per c.c. in the Samples of Milk
1	55	9	16.4	10 50,000
2	40	2	5.0	3200 4000
3	28	7	25.0	180 680
4	32	8	25.0	50 3000
5	37	19	51.4	40 7000

Frequency of the B. Abortus Group in Freshly Drawn Milk.—In discussing the frequency of this group in samples of milk, it is necessary to emphasize the fact that the figures presented in Table 8 are undoubtedly far below the actual numbers present in the milk. Particularly is this true in regard to the figures for Dairies 1 and 2, since the method described for determining the presence of the B. abortus type was developed gradually as the work progressed, and was not perfected until the last series of plates was made, for Dairy 5. The

figures may be regarded with increasing accuracy, therefore, from Dairy 1 to Dairy 5. Table 8 shows that bacteria of the B. abortus type occurred commonly in the samples of milk from all 5 of the dairies. Altogether, cultures were isolated from 45, or 23.4% of the 192 samples studied.

Description of the Varieties of B. Abortus from the Udder.—About one-half of the cultures of B. abortus isolated from the milk samples were identical in their behavior towards every medium into which they were inoculated. The most vigorous activity of these cultures was found in their hydrolization of butter fat. No description of this variety can be found in the literature. In fact, no recognition of the different varieties of B. abortus has been found. Therefore, the name B. abortus var. lipolyticus is given to the fat-splitting variety, to distinguish these cultures from those which do not have the fat-splitting property. A description of any one of the 33 cultures of this variety would apply equally well to all the others.

The cultures of B. abortus var. lipolyticus produce a very faint growth on meat-infusion agar slopes. The growth is in very small separate colonies, which are scattered over the whole surface of the slope if it happens to be moist at the time of inoculation, or the colonies are confined to a ribbon-like growth along the line of inoculation if the agar is comparatively dry.

Five percent of bile added to the agar gives a more vigorous growth. Ten percent of blood serum added to the agar gives the most favorable conditions for growth. On this medium the growth is vigorous and fairly rapid and in a few days the medium begins to take on a brownish tinge, which deepens with the age of the culture.

The lipolyticus variety illustrates admirably the peculiar preference of B. abortus for an oxygen supply at less than atmospheric pressure, which led Nowak[26] to devise special methods for incubating his cultures with the optimal conditions of aeration. When agar-shake cultures are seeded heavily throughout the entire mass, growth takes place in a thin layer, a few millimeters beneath the surface.

The cultures of B. abortus var. lipolyticus produce no effect on gelatin, nitrates, urea, or asparagin; they produce no acid from any of the carbohydrates and related substances which are commonly used for fermentation tests. In litmus milk from which the cream has been removed the growth is slight, and there is no apparent effect. But in litmus whole milk there is a rapid multiplication of cells, and slow development of acid, which is first evident in the cream layer. After a couple of weeks' growth at 37 C., the optimal temperature, the cream layer is converted into a firm, slightly granular, ill-smelling mass.

Cultures of the lipolyticus type were isolated from 33, or 12% of the 192 samples studied. ·This type was isolated from samples of milk taken from all 5 of the herds.

Seven other cultures were considered to be atypical B. abortus var. lipolyticus because they differed from the 33 type cultures in forming a heavier growth on the infusion-agar slope, which was of an apricot yellow color (Ridgway's "Color Standards and Nomenclature" Plate 19, YOY-b). Also, 4 of these cultures decomposed urea.

Another group of bacilli isolated from the samples of milk was considered a variety of B. abortus and is designated Variety *b*. These bacilli were similar

in morphology to all those included in the B. abortus type. Dextrose and maltose broths were rendered acid. There was no change of reaction in the other broths. This variety failed to break down butter fat. Growth on plain infusion agar was abundant and of a dirty yellowish color. In agar shake cultures growth occurred on the surface and in the upper 6 to 10 millimeters of the agar. The "diaphragm" growth rarely occurred. Asparagin, nitrates, and urea were usually decomposed. Gelatin was not liquefied. Since growth was vigorous on plain infusion agar, it was not especially favored by the addition of serum. Twelve cultures of this variety were isolated from Dairies 1, 3, and 5.

Still another variety, designated *c,* differed from Variety *b* only in rendering the broth media slightly alkaline and in liquefying gelatin in the case of a few cultures. Only 5 cultures of this variety, all from Dairy 5, were isolated.

Relationship Between the Varieties of the B. Abortus Group Found in Milk and Those From Pathogenic Sources.—It was a difficult problem to classify these bacilli common in milk from normal udders, for no complete descriptions of these varieties could be found. A few striking characteristics possessed in common with the bacillus of contagious abortion as described in the literature suggested the relationtionship to those organisms and a thorough study of the cultures from milk was made in order to compare them with the original descriptions of B. abortus, and with cultures obtained from pathogenic sources. The problem thus led to a study of the serum reactions of the bacilli from milk in comparison with those of cultures from pathogenic sources, and to a study of the pathogenic properties of the cultures from milk. These phases of the problem are being worked out in co-operation with the pathological division of this bureau and will be the subject of a separate paper.

A comparison of the organisms of this group, so far as the bacteriologic methods would go, is presented in a briefly summarized form in Table 9. In the 1st column is given a description of B. abortus found in the literature on the subject. The description given by Bang,[3] who first described the organism in 1897, supplied most of the data for this column. Nowak's[26] description of B. abortus is quoted under two headings where Bang's description was lacking. The descriptions of these two investigators fully agree. In the 2nd column is given a description in summary of 24 cultures from pathogenic sources which were supplied by the pathological division of this bureau for this purpose. The cultures had been collected originally by several institutions in different states, and had all been under artificial cultivation for several months before the present study was made.

TABLE 9

A COMPARISON OF THE CHARACTERISTICS OF THE SEVERAL VARIETIES OF BACILLUS ABORTUS

	B. Abortus from Original Descriptions	B. Abortus from Pathogenic Sources	B. Abortus lipolyticus	B. Abortus Variety b	B. Abortus Variety c
Morphology	Small rods, the largest as long as the tubercle bacilli (Bang)	Slender rods, 0.8 to 1.5 microns in length	Slender rods 0.8 to 1.5 microns in length	Slender rods 0.8 to 1.5 microns in length	Slender rods 0.8 to 1.5 microns in length
Reaction in dextrose, maltose, lactose, raffinose, mannite, and glycerin broth	Alkaline broth is given an amphoteric or slightly acid reaction to Tournesol paper (Nowak)	Neutral broth is rendered slightly alkaline, except that a few cultures form a slight acidity in dextrose broth	No change......	Dextrose and maltose broths are rendered acid. No change in other brotbs	Slightly alkaline
Decomposition of nitrogenous compounds	....	Nitrate, asparagin, and urea are commonly decomposed. Gelatin is not liquefied	Asparagin and nitrates are not decomposed. Urea is rarely decomposed. Gelatin is not liquefied	Asparagin nitrates and urea are usually decomposed. Gelatin is not liquefied	Asparagin, nitrates, and sometimes urea are decomposed. Gelatin is sometimes liquefied
Action in litmus whole milk	....	Rendered slightly alkaline	Acid is developed in the cream layer	In most cases, slightly alka-.line. Sometimes no change	No change
Growth in agar shake	Growth in colonies is confined to a zone of from 10 to 15 mm. This zone lies about 5 mm. beneath the surface of the agar (Bang)	Good growth on surface. Sometimes a growth throughout a zone of several millimeters at the top. Rarely a "diaphragm" growth	Colonies confined to a thin layer a few millimeters beneath the surface	Like the growth of cultures from pathogenic sources. Sometimes colonies are scattered throughout the entire depth of the agar	Like the growth of cultures from pathogenic sources
Growth on plain infusion-agar slope	Separate colonies resemble rose-colored droplets reflecting a greenish tinge (Nowak)	Abundant compact growth. Color, 19″YOY, b and d (chamois and cream buff)	A few cultures resemble those from pathogenic sources. The majority give a scant growth in separate colonies	Like the growth of cultures from pathogenic sources	Like the growth of cultures from pathogenic sources
Growth in glycerin broth	A poor growth. A fine sediment is thrown down, made up of whitish grains (Bang)	Good growth which clouds the medium	Scant growth which does not cloud the medium. Sediment is made up of little granules	Abundant growth. The medium is usually clouded, but sometimes all growth is precipitated, leaving a clear medium	Like the growth of Variety "B"
Effect of serum in the agar	Growth is greatly favored (Bang)	Abundant growth without the serum	Growth greatly favored	Abundant growth without the serum	Abundant growth without the serum

The table shows that altho the morphology is fairly constant there is a wide variation in the varieties described under the different columns. There appears to be an essential difference between the characteristics of the organisms described by Bank and Nowak and those of the cultures collected from pathogenic sources in this country. Such differences can scarcely be accounted for unless it be assumed that the European investigators were working with another variety of the abortion bacillus.

The characteristics of the lipolyticus variety, in the 3rd column, agree in every respect with Nowak's description except that the color on agar slopes was not observed in the case of the lipolyticus variety. Most striking is the similarity of the peculiar growth of the lipolyticus variety in agar-shake cultures to that of Bang's bacillus. The colonies develop only in a thin layer a few millimeters below the surface of the agar. Bang identified B. abortus in mixed cultures by this one characteristic.

On the other hand, Varieties *b* and *c* resemble the cultures studied from pathogenic sources. Variety *b* differs from those cultures chiefly in its reaction in the different broths. Variety *c* bears a still greater resemblance to the cultures from pathogenic sources but 3 of the 5 cultures liquefied gelatin, and 2 of these 3 digested milk slowly.

Significance of the B. Abortus Type in Milk.—Aside from the interest due to an organism which is commonly present in milk and which is closely related to the pathogenic B. abortus, this type is important because of its biochemical activities. The ability of the cultures of the lipolyticus variety to form acid in whole milk has been mentioned. Since no acid was formed in milk from which the cream had been removed, it was reasonable to suppose that the acid was formed by the breaking down of the butter fat. Accordingly chemical determinations of the volatile acids formed in milk by cultures of the acid-producing type were made. (Chemical analyses were made by Dr. J. N. Currie, of the dairy division.) Culture *qj* incubated for 15 days in 375 c.c. of 20% cream on distillation to 1000 c.c. with steam gave 11.02 n/10 c.c. more of volatile acids than did the control flask.

Five other cultures were grown in 750 c.c. whole milk for periods varying from 2 to 3 weeks. The cultures were made slightly acid with dilute sulfuric acid and distilled with steam until 2000 c.c. of distillate were collected. The volatile acids contained in the distillates were estimated by a Duclaux distillation. The figures obtained and also the average figures for two controls are given in Table 10.

The figures show that all but one of the cultures formed volatile acids. It is a significant fact that butyric and caproic acids were found to be present in approximately the proportion in which they occur in butter fat.[6] This, together with the fact that the more active cultures gave very considerable quantities of insoluble acids, shows conclusively that this group of organisms can cause hydrolysis of the fat.

A small amount of acid of lower molecular weight than butyric was present in all the cultures. This has been reported as formic acid. Hart, Hastings, Flint, and Evans[20] found from 11.763 to 12.567 decinormal cubic centimeters of volatile acids in 300 c.c. of sterile milk incubated 4 months at 37 C. They reported almost equivalent quantities of acetic and formic acids. Mixtures of these two acids so small in quantity as the figures in our experiments show, could not be identified with much certainty by the process of distillation employed. The

TABLE   10

VOLATILE ACIDS FROM 750 C.C. OF WHOLE MILK INOCULATED WITH B. ABORTUS VAR. LIPOLYTICUS.   RESULTS EXPRESSED IN N/10 C.C.

Acid	me	hw	np	nj	nw	Control
Caproic....................	7.22	2.77	2.18	0.78	0.00	0.00
Butyric....................	13.74	6.65	5.90	2.36	1.06	1.01
Formic.....................	3.62	11.74	2.95	4.69	3.11	4.35
Insoluble..................	3.73	0.50	0.00	0.00	0.00	0.00

cultures with one exception, *hw*, show nearly the same quantity of formic acid as the control. This would indicate that the acid was probably not produced by the bacteria but by some reaction during the sterilization or distillation. Therefore no special significance is attached to the formic acid figure.

The figures show in most cases only small amounts of butyric acid, and it required a growth of 2 weeks or more under optimal conditions of temperature to develop these small amounts. But since butyric acid possesses a very offensive odor, which can be detected in minute proportions of the acid, it was thought that perhaps the B. abortus group might have something to do with the development of undesirable flavors and odors in ripening cream. An earlier experiment had shown that lactic acid added to the milk to bring its acidity to 0.4% had no effect upon the multiplication of these organisms in the cream layer. This indicated that the by-products of organisms normally present in cream in large numbers do not check the growth of B. abortus.

A practical experiment was conducted to determine whether B. abortus var. lipolyticus might render cream undesirable under conditions similar to those to which cream is often subjected before use. The conditions were exaggerated in regard to the number of B. abortus present at the beginning of the experiment, altho on one or two occasions these organisms have been isolated from milk in numbers comparatively as high.

A very good quality of cream was mixed and then divided into 3 equal portions of 1 pint each. One flask of cream served as a control, and the other 2 were inoculated with cultures of the B. abortus type which had been isolated from milk. Culture *mc* used to inoculate one flask, was a typical culture of the lipolyticus variety.

Culture *sr*, which was used to inoculate the other flask, had been isolated from a "Special Milk" purchased in the market and supposed to be a good grade of raw milk. The lactose-agar plates made from this sample showed 78,000 colonies. The serum-agar plates showed 570,000 colonies per cubic centimeter. This indicated almost 500,000 of the B. abortus group per cubic centimeter. Culture *sr*, which represented these colonies, resembled the B. abortus type in morphology and general behavior. The manner of growth in serum agar, failure of growth in lactose agar, its morphology, and the fact that it was active in decomposing butter fat, indicated that it was related to the B. abortus type, and the finding of this strain in such large numbers in market milk gave a special interest to this organism in the experiment here recorded.

The three flasks of cream were allowed to stand in the room for about an hour, and then were placed in a thermostat at 17 C. where they were kept for 2 days, or until the end of the experiment. But they were removed twice to room temperature for a half hour, and once for 2 hours. This was to simulate the conditions to which cream might be subjected in a kitchen where no especial attention was given to its care.

TABLE 11

Effect of B. Abortus on Flavor of Cream

Judge	I Control Cream	II Cream Inoculated with MC	III Cream Inoculated with SR
		24 Hours after Inoculation	
A	Smoother in texture than others	Disagreeable odor. Taste not affected	Same as II
B	Good cream.........	An old, tho not bad, flavor	Same as I
C	Good cream.........	Slightly bad odor, but no difference in flavor	Same as II
D	Good cream.........	Bad odor, no difference in flavor	Same as I
E	Perfectly clean flavor and odor	Bad odor and taste.......	Worse flavor than II
		48 Hours after Inoculation	
B	Mild odor............	Bad odor, no difference in flavor	Same as II
C	By far the best.....	The worst of the three in odor. Flavor better than III	The worst of the three in taste, but odor not so bad as II
E	The best of the three	Not a good flavor........	The worst of the three

Twenty-four hours after inoculation and again 48 hours after inoculation the creams were submitted to the judgment of several persons who knew nothing of the experiment. The flasks were relabeled before the second scoring. The opinions of the judges are arranged in tabular form in Table 11, which shows that after 24 hours the flask inoculated with Culture *mc,* which is typical of the strains isolated most frequently from milk, had acquired a disagreeable flavor and odor which was detected by all 5 judges. The flask inoculated with Culture *sr,* from market milk, was apparently not so bad, for only 3 of the 5 judges detected anything wrong with the cream. At the time of the 1st scoring all the creams were sweet. At the time of the 2nd scoring all 3 of the creams had soured. The creams which had been inoculated had large clots which could not be broken by violent shaking, and there had been a decided deterioration in the quality.

TABLE   12

Bacterial Content of Cream Inoculated with Bacillus Abortus

	Control Cream		Cream Inoculated with MC		Cream Inoculated with SR	
	Total Number of Bacteria per c.c.	Number of B. Abortus per c.c.	Total Number of Bacteria per c.c.	Number of B.Abortus per c.c.	Total Number of Bacteria per c.c.	Number of B. Abortus per c.c.
At time of inoculation..	15,000	None isolated	280,000	265,000	1,500,000	1,200,000
After 24 hours...........	8,200,000	None isolated	12,500,000	2,000,000	12,000,000	None isolated
After 48 hours...........	540,000,000	None isolated	740,000,000	200,000,000	650,000,000	None isolated

Bacteriologic analyses of the creams just after inoculation and at the times of judging were made, the figures of which are given in Table 12. The figures showing the number of B. abortus are only rough approximations. The control cream had a low bacterial content, and if it contained B. abortus the number was so small that they failed to be isolated. The rapid multiplication of *mc,* in the presence of the normal flora of cream, shows what a previous experiment had indicated; namely, that the growth of B. abortus is not checked by the by-products of the other bacteria normally present in milk and cream. It shows also that B. abortus multiplies rapidly in cream that is subjected to temperatures at which it is commonly kept before use.

The inoculation of Culture *sr* was very heavy, but the other bacteria multiplied so much more rapidly that it failed to be isolated in the 2nd and 3rd analyses. There can be no doubt, however, that it exerted a deteriorating effect on the cream, for the appearance, odor, and flavor were affected, particularly on the second day, when this cream was unquestionably inferior to the control.

The results of this experiment show that B. abortus may be the cause of bad odors and flavors in ripened cream. The odor and flavor obtained after inoculation were such as are common in old cream.

B. Abortus in Mixed Market Milk.—Since B. abortus was found in samples of milk from individual cows of all 5 herds from which samples were taken, it was to be expected that these organisms could be isolated from mixed market milk, with low bacterial content. If the

bacterial content is very high, there might be large numbers of B.
abortus present, but they would fail to be isolated. Therefore, the
mixed market milks examined, were ascertained to be of good quality.
The finding of organisms represented by Culture *sr* in numbers of
about 500,000 per cubic centimeter in a good grade of market milk
has already been mentioned.   No B. abortus could be isolated from
the cream used in the experiment recorded here, but it had been
isolated 2 weeks earlier from mixed whole milk produced at the farm
from which this cream came.   Fewer than 200 abortion bacilli per
cubic centimeter appeared in this milk.

Two bottles of certified milk were purchased in market at Chicago,
kept at room temperature for about 6 hours, and then plated on serum
agar. About 28,000 of the abortion bacilli were shown to be present
in the cream layer of one sample and about 1900 in the cream layer
of the other sample.   In both samples the number of B. abortus was
about 25% of the whole number of bacteria.   The abortion bacilli can
therefore be demonstrated in mixed milk.

Persistence of B. Abortus in Cows' Udders.—The milks of 4 of the
cows of Dairy 1, which had contained B. abortus, were examined a
2nd time more than a year after the 1st examination.   The samples
were taken from the same quarter of the udder for both examinations.
The data are given in Table 13.   In the case of the two cows which
gave milk containing only a few abortion bacilli at the time of the 1st
examination, none of these organisms could be isolated from the milk
in the 2nd examination.   Cow 201, which gave 50,000 abortion bacilli
per cubic centimeter at the time of the 1st examination, continued to
give them in very large numbers.   Cow 204 also continued to give these
organisms in her milk in considerable numbers.

CONCLUSIONS

It appears from the study here reported that there is a definite
udder flora comprising bacteria which belong to parasitic types.
It is not surprising that the majority of udder bacteria should be of
the same type as those common on the skin and mucous membrane of
man and animals.   The majority of the staphylococci on the skin are
of the non-virulent variety which fails to produce pigment and fails
to ferment mannite.   But pathogenic varieties also are found on the
skin, where they ordinarily cause no trouble.   Similarly, the majority
of the staphylococci of the udder are non-virulent, but varieties which

are capable of causing death when inoculated into experimental animals occasionally establish themselves in healthy udders. Whatever the variety may be, conditions in the udder are favorable to multiplication, and frequently large numbers are eliminated in the milk.

The pathogenic properties of the streptococci and bacilli common in milk when it leaves the udder are not discussed in this paper, but they, also, are parasitic in their nature.

When a bacterial culture is tested for its pathogenic properties, the body tissues and fluids are exposed directly to the toxins of the culture in question. When organisms enter into the digestive tract with the food the circumstances are different, for the body tissues and fluids are protected by the mucous membranes against the ravages of the bacteria that enter with the food. Therefore it cannot be

TABLE 13
PERSISTENCE OF B. ABORTUS IN COWS' UDDERS

Cow	Quarter	First Examination		Time Between 1st and 2nd Examinations	Second Examination	
		Total Bacterial Count per c.c.	Total B. abortus per c.c.		Total Bacterial Count per c.c.	Total B. abortus per c.c.
14	RB	750	Estimation impossible	19 months	230	None
18	RF	130	50	13 months	50	None
201	RF	50,000	50,000	13 months	10,000	10,000
204	RF	5600	5000	13 months	850	850

assumed that bacteria which are pathogenic to inoculated laboratory animals would be injurious to human beings when present in the milk consumed. It is a subject worthy of investigation. But since the bacteria of the udder are parasitic in their nature, and since pathogenic varieties are sometimes eliminated in considerable numbers from healthy udders, the data here reported add evidence to the growing conviction that all milk is safer for consumption after it has been pasteurized.

SUMMARY

One hundred and ninety-two samples of milk from 161 cows of 5 different dairies in 2 widely distant sections of the country have been studied. No consideration was given to those types of bacteria which occurred in the udder in small numbers. All the cultures which were studied in detail and included in this report represent bacteria which were multiplying in the udder and were found in the milk in considerable numbers.

TABLE 14

CHARACTERISTICS OF THE STAPHYLOCOCCI IN FRESHLY DRAWN MILK

Culture	Dairy	Cow	Action in Milk	Dextrose	Lactose	Maltose	Raffinose	Mannite
			GROUP A					
ab	1	1	Curdled in 4 days	4.60	3.65	3.45	0.30	0.75
ak	1	2	Curdled in 4 days	4.75	4.35	2.85	0.20	1.35
cg	1	23	No change in 14 days	3.50	3.35	3.40	0.40	0.80
cn	1	23	Acid, not curdled, in 14 days..	1.10	1.05	1.60	0.25	0.65
cq	1	25	Curdled in 5 days	2.60	2.10	2.55	0.40	0.85
cs	1	100	Acid, not curdled, in 14 days..	3.25	3.15	3.20	−0.05	0.85
dc	1	27	Acid, not curdled, in 14 days..	2.75	3.30	2.55	0.35	1.25
dq	1	13	Acid, not curdled, in 14 days..	4.30	3.15	3.45	0.15	1.30
dr	1	13	Acid, not curdled, in 14 days..	4.70	3.60	3.50	0.10	1.25
ds	1	14	Curdled in 5 days	5.00	4.90	3.15	0.15	1.05
ea	1	7	Curdled in 14 days	3.75	4.30	3.05	0.05	1.25
fj	1	204	Acid, not curdled, in 14 days..	4.05	3.80	3.25	0.15	1.35
in	1	27	Curdled in 10 days	4.20	5.00	2.90	0.05	1.35
is	1	204	No change in 14 days	3.65	3.75	3.05	0.05	0.75
jm	1	2	Slight acidity in 14 days	3.55	3.25	0.70	0.10	1.00
kb	1	12	Curdled in 11 days	4.00	3.70	1.65	0.05	0.90
kn	1	12	Curdled in 11 days	4.05	4.00	1.75	0.15	2.05
kz	2	10051	Acid, not curdled, in 14 days..	3.15	3.50	4.10	0.05	1.15
lb	2	10039	Acid, not curdled, in 14 days..	3.60	3.25	3.80	0.06	1.15
lm	2	10006	Acid, not curdled, in 14 days..	3.25	3.55	3.95	0.10	0.60
lx	2	14759	Acid, not curdled, in 14 days..	3.95	3.45	2.20	0.10	1.30
mj	3	152	No change in 14 days	3.85	2.70	3.10	0.05	1.25
ml	2	52	No change in 14 days	3.20	1.85	2.45	0.15	1.50
nf	4	304	Acid, not curdled, in 14 days..	3.75	3.50	3.25	0.10	1.30
nh	4	319	Curdled in 4 days	3.25	2.30	2.90	0.20	1.85
ni	4	330	Curdled in 4 days	3.75	3.00	2.80	0.25	1.60
ol	5	8	Acid, not curdled, in 14 days..	1.10	3.85	1.85	−0.20	0.80
ou	5	11	Acid, not curdled, in 14 days..	1.00	2.80	1.90	0.15	1.75
px	5	30	Acid, not curdled, in 14 days..	4.00	3.20	2.45	0.00	1.00
pz	5	31	No change in 14 days	3.85	3.00	2.30	0.00	1.55
qq	5	39	Acid, not curdled, in 14 days..	3.15	3.35	2.50	−0.15	0.85
qy	5	42	No change in 14 days	3.05	2.75	2.30	−0.15	1.35
rf	5	53	No change in 14 days	4.20	3.55	2.25	0.00	1.25
rj	5	55	Acid, not curdled, in 14 days..	4.60	3.70	2.15	−0.45	1.20
rq	5	56	Acid, not curdled, in 14 days..	4.20	3.55	2.50	−0.20	1.50
rv	5	58	Acid, not curdled, in 14 days..	3.40	2.95	2.35	0.20	0.85
rz	5	59	Acid, not curdled, in 14 days..	4.25	3.80	1.55	−0.10	1.75
			GROUP B					
an	1	2	Curdled in 4 days	4.00	2.00	3.25	0.30	0.90
ap	1	2	Curdled in 2 days	3.60	1.70	3.30	0.35	1.50
bn	1	3	Curdled in 4 days	4.90	1.85	3.20	0.25	1.65
dx	1	12	Acid, not curdled, in 14 days..	3.90	3.90	3.30	0.40	2.25
dy	1	4	No change in 14 days	3.80	3.50	3.45	0.00	1.10
eb	1	9	Curdled in 4 days	4.00	4.20	3.65	0.20	3.20
fd	1	16	Acid, not curdled, in 14 days..	4.15	4.15	3.15	−0.05	1.30
fe	1	16	Acid, not curdled, in 14 days..	3.65	3.80	3.65	0.10	2.25
fg	1	21	Acid, not curdled, in 14 days..	4.30	4.30	2.90	0.00	1.15
fl	1	205	Acid, not curdled, in 14 days..	4.65	3.90	3.55	0.10	1.05
fs	1	21	Acid, not curdled, in 14 days..	3.20	1.15	3.50	0.10	1.35
fw	1	24	Acid, not curdled, in 14 days..	3.25	2.90	2.75	0.25	1.00
ia	1	16	Acid, not curdled, in 14 days..	3.40	4.10	3.15	−0.15	3.25
ib	1	16	Acid, not curdled, in 14 days..	3.30	3.10	4.20	0.10	3.10
ij	1	21	Curdled in 4 days	3.40	2.40	1.40	0.25	1.10
im	1	27	Curdled in 10 days	3.75	4.55	3.20	−0.10	1.30
io	1	27	Curdled in 10 days	4.20	5.90	2.60	0.10	1.65
ip	1	27	Curdled in 10 days	3.90	3.45	2.65	0.05	1.55
jo	1	2	Curdled in 11 days	3.35	2.85	1.80	0.25	0.80
nq	4	289	Curdled in 4 days	3.25	3.20	4.15	0.40	1.85
rk	5	55	Curdled in 10 days	5.65	3.20	2.45	−0.40	2.25
rr	5	56	Curdled in 14 days	3.10	3.20	2.20	−0.15	1.95
so	5	59	Curdled in 14 days	3.35	3.05	2.05	−0.05	1.75
sf	5	68	Curdled in 14 days	3.50	3.30	1.65	−0.10	1.35
sm	5	75	Curdled in 14 days	3.25	3.00	2.05	0.00	2.10

TABLE 14—*Continued*

Characteristics of the Staphylococci in Freshly Drawn Milk

Glycerin	Hemolysis	Liquefaction of Gelatin	Reduction of Nitrates	Decomposition of Asparagin	Decomposition of Urea	Chromogensis
			Group A—*Continued*			
0.10	+++	30 mm.	--	--	+	19 YOY, light cadmium
1.35	+++	20 mm.	—	—	+	19 OY d, buff yellow
0.45	+++	8 mm.	---	—	+	19 YOY, light cadmium
0.20	—	6 mm.	--		—	Slight pigment
0.35	+	6 mm.	—	—	+	17 OY f, pale yellow orange
0.30	+++	10 mm.	--	—	+	19 YOY, light cadmium
0.30	—	0 mm.	—	—	--	23″ Y f, marguerite yellow
0.45	+++++	11 mm.	—	--	+	17 OY, cadmium yellow
0.30	+++	8 mm.	—		+	17 OY, cadmium yellow
0.45	+++	18 mm.	—	—	+	19 YOY d, buff yellow
0.45	+++	17 mm.	—		+	19 YOY, light cadmium
0.25	+++	13 mm.	—	--	+	17 OY, cadmium yellow
0.35	+++	17 mm.	—	—	—	19 YOY b, apricot yellow
-0.05	+++	Slight	—	—	—	17 OY, cadmium yellow
0.15	+++	6 mm.	—	—	—	19 YOY b, apricot yellow
0.20	+++	18 mm.	—	—		19 YOY, light cadmium
0.20	+++	16 mm.	—	—	--	19 YOY b, apricot yellow
0.05	+++	0 mm.	—	—	+	17 OY, cadmium yellow
0.40	+++	0 mm.	—	—	+	17 OY, cadmium yellow
0.30	+++	0 mm.	—	—	+	19 YOY b, apricot yellow
0.25	+++	0 mm.	—	—	+	17 OY b, deep chrome
0.35	++	8 mm.	—		+	15 YO, orange
0.45	+	0 mm.	—	—	+	17 OY, cadmium yellow
0.30	+++	0 mm.	--	—	+	17 OY b, deep chrome
0.20	++++	20 mm.	—	—	+	17 OY b, deep chrome
0.25	++++	28 mm.	—	—	—	17 OY, cadmium yellow
0.20	+++	25 mm.	—	—	--	17 OY, cadmium yellow
0.45	—	24 mm.	+	—	+	19″ YOY f, cartridge buff
0.45	+++	25 mm.	-	—	--	17 OY, cadmium yellow
0.30	++	10 mm.	—	—	+	19 YOY, light cadmium
0.25	+	15 mm.	+	—	+	15 YO d, orange buff
0.35	+++	0 mm.	--	--	+	15 YO d, orange buff
0.35	+	15 mm.	—	—	⊥	17 OY b, deep chrome
0.30	—	27 mm.	--	—	—	17 OY, cadmium yellow
0.45	+++	27 mm.	—	—	—	17 OY, cadmium yellow
0.30	+	0 mm.	—	—	+	17 OY f, pale orange yellow
0.40	+++	0 mm.	—	—	—	17′ OY b, antimony yellow
			Group B—*Continued*			
1.15	÷	12 mm.	—	—	+	17 OY b, deep chrome
1.80	—	7 mm.	—	—	+	15 YO b, capucine yellow
0.90	+	16 mm.	+	—	+	19 YOY f, maize yellow
0.65	++++	20 mm.	—	—	+	17 OY b, deep chrome
0.65	+++	28 mm.	--	—	+	17 OY, cadmium yellow
1.55	+++	21 mm.	—	—	+	17 OY, cadmium yellow
0.60	+++	17 mm.	—	—	+	17 OY b, deep chrome
1.10	+++	16 mm.	—	—	+	19″ YOY f, cream color
0.60	+++	14 mm.	—	—	+	19 YOY, light cadmium
0.55	+++	15 mm.	—	—	+	15 YOY d, orange
0.70	—	15 mm.	—	—	+	19 YOY d, buff yellow
0.80	—	10 mm.	—		+	19 YOY d, buff yellow
1.05	++++	16 mm.	+	—	+	19″ YOY, honey yellow
1.70	++++	27 mm.	+	—	+	19″ YOY, honey yellow
0.75	++	15 mm.	+		+	15 YO b, capucine yellow
0.70	+++	20 mm.	—	—	—	17 OY b, deep chrome
0.55	+++	16 mm.	—	—	—	19 YOY d, buff yellow
0.60	+++	18 mm.	--	—	—	17 OY b, deep chrome
0.60	+++	14 mm.	—	—	—	19 YOY, light cadmium
0.85+	+++	25 mm.	—	—	+	17 OY b, deep chrome
0.90	+++	31 mm.	—	—	+	15 YO d, orange buff
0.70	—	18 mm.	—	—	—	19″ YOY f, cartridge buff
1.65	—	20 mm.	—			19″ YOY f, cartridge buff
0.70	—	25 mm.	—	—	—	19″ YOY f, cartridge buff
2.20	—	25 mm.	+	—	—	19″ YOY f, cartridge buff

TABLE 14—*Continued*

CHARACTERISTICS OF THE STAPHYLOCOCCI IN FRESHLY DRAWN MILK

Culture	Dairy	Cow	Action in Milk	Dextrose	Lactose	Maltose	Raffinose	Mannite
			GROUP C					
ac	1	1	Curdled in 5 days.............	4.20	1.20	2.95	0.50	0.90
ar	1	3	Curdled in 5 days.............	3.65	2.00	2.65	0.50	1.20
bb	1	2	Curdled in 4 days.............	3.75	2.30	3.05	0.75	1.30
be	1	2	Curdled in 5 days.............	4.95	4.20	2.15	0.55	1.20
bw	1	23	Curdled in 5 days.............	4.00	1.90	3.25	0.65	1.85
ck	1	21	Curdled in 4 days.............	2.60	1.55	2.25	0.65	2.35
cl	1	21	Acid, not curdled, in 14 days..	2.15	3.80	2.80	1.70	1.20
cx	1	19	Acid, not curdled, in 14 days..	1.95	2.35	2.90	1.20	1.15
da	1	26	Acid, not curdled, in 14 days..	1.40	2.15	3.15	0.55	0.55
oi	5	1	Acid, not curdled, in 14 days..	1.45	2.40	2.35	1.15	0.90
op	5	9	Acid, not curdled, in 14 days..	2.85	0.75	2.15	1.00	0.90
po	5	23	Curdled in 5 days.............	1.60	3.10	2.50	1.35	2.05
			GROUP D					
aj	1	2	Curdled in 4 days.............	3.75	1.50	3.30	0.85	1.25
az	1	2	Curdled in 4 days.............	4.40	1.55	2.89	0.80	1.35
ba	1	2	Curdled in 5 days.............	3.55	1.35	2.60	0.55	1.30
bd	1	1	Curdled in 4 days.............	4.50	1.05	2.70	0.50	0.60
bo	1	3	Curdled in 4 days.............	1.95	2.25	3.05	0.65	1.40
bx	1	23	Curdled in 5 days.............	2.95	1.85	2.90	0.50	1.40
cc	1	100	Curdled in 5 days.............	2.65	2.05	2.89	1.40	0.65
cd	1	100	Curdled in 5 days.............	2.95	2.45	3.05	1.00	1.50
ci	1	17	Curdled in 5 days.............	2.90	2.15	2.55	0.90	1.45
cm	1	21	Curdled in 5 days.............	3.90	1.45	3.15	0.90	1.75
co	1	23	Curdled in 5 days.............	2.75	1.00	2.95	0.75	1.30
cr	1	27	Acid, not curdled, in 14 days..	1.40	2.05	2.90	1.00	1.15
ct	1	100	Curdled in 5 days.............	2.50	1.70	3.20	0.75	1.80
df	1	19	Acid, not curdled, in 14 days..	1.85	1.80	0.85	1.35	2.10
dm	1	7	Acid, not curdled, in 14 days..	4.85	4.70	2.70	0.50	1.35
nl	4	62	No change in 14 days.........	2.95	1.60	2.20	1.45	1.30
of	5	1	No change in 14 days.........	3.15	2.90	1.95	1.85	1.35
qv	5	39	Curdled in 10 days.............	3.55	2.40	2.15	1.05	1.50
rb	5	46	Acid, not curdled, in 14 days..	2.85	2.20	2.35	0.80	0.95
ru	5	58	Acid, not curdled, in 14 days..	1.05	2.90	2.20	2.45	0.65
			GROUP E					
nx	4	160	Acid, not curdled, in 14 days..	5.45	3.10	2.05	3.90	0.05
oh	5	1	Acid, not curdled, in 14 days..	1.25	3.25	3.05	1.40	0.05
			GROUP F					
aa	1	1	Curdled in 2 days.............	5.20	4.20	4.50	0.35	0.00
am	1	2	Acid, not curdled, in 14 days..	5.25	3.70	5.20	0.20	—0.40
at	1	14	Curdled in 2 days.............	4.60	3.95	4.10	0.20	0.25
aw	1	14	Curdled in 4 days.............	4.85	4.05	4.15	0.35	0.15
ax	1	14	Curdled in 2 days.............	4.75	3.80	4.30	0.40	—0.05
ay	1	2	Curdled in 4 days.............	5.20·	4.60	4.45	0.25	—0.20
bh	1	1	Curdled in 4 days.............	4.45	4.25	4.35	0.20	—0.15
bi	1	14	Curdled in 4 days.............	4.90	3.60	4.24	0.25	0.05
du	1	9	Curdled in 5 days.............	4.30	4.50	4.20	0.05	0.20
dv	1	13	Curdled in 7 days.............	4.80	4.40	4.05	0.10	0.30
ef	1	9	Curdled in 6 days.............	4.40	3.90	3.90	0.25	0.15
eh	1	10	Curdled in 6 days.............	3.65	4.00	4.15	0.15	0.20
ej	1	13	Curdled in 6 days.............	4.30	3.80	4.10	0.25	—0.15
fq	1	19	Curdled in 6 days.............	4.35	3.90	4.00	0.10	—0.05
fr	1	19	Curdled in 7 days.............	4.35	3.60	4.00	0.15	—0.15
jn	1	2	Curdled in 8 days.............	4.50	4.10	3.25	0.10	0.15
jr	1	8	Curdled in 6 days.............	3.45	3.45	3.90	0.00	—0.05

TABLE 14—*Continued*

CHARACTERISTICS OF THE STAPHYLOCOCCI IN FRESHLY DRAWN MILK

Glyc-erin	Hemoly-sis	Lique-faction of Gelatin	Reduc-tion of Nitrates	Decom-position of As-paragin	Decom-position of Urea	Chromogensis
			GROUP C—*Continued*			
0.15	+	10 mm.	+	—	+	17 OY f, pale orange yellow
0.25	+++	Slight	+	—	+	15 YO b, capucine yellow
0.50	++	Slight	+	—	+	15 YO b, capucine yellow
0.35	+++	6 mm.	—	—	+	19 YOY d, buff yellow
0.50	+	15 mm.	—	—	+	17 OY d, light orange yellow
0.45	+++	12 mm.	—	—	+	15 YO f, pale yellow orange
0.45	—	Slight	—	—	+	17 OY f, pale orange yellow
0.30	—	0 mm.	—	—	—	19 YOY, light cadmium
0.35	+	2 mm.	—	—	+	19 YOY d, buff yellow
0.40	+	12 mm.	—	—	+	19 YOY d, buff yellow
0.15	—	12 mm.	—	—	+	15 YO d, orange buff
0.40	++++	25 mm.	—	—	+	17″ OY b, antimony yellow
			GROUP D—*Continued*			
1.45	++	12 mm.	+	—	+	15 YO b, capucine yellow
0.75	+	8 mm.	+	—	+	15 YO b, capucine yellow
0.65	+	10 mm.	—	—	+	15 YO b, capucine yellow
0.60	+	Slight	—	—	+	15 YO d, orange buff
0.55	—	Slight	+	—	+	15 YO b, capucine yellow
0.80	+	9 mm.	+	—	+	17 OY b, deep chrome
0.55	+	15 mm.	—	—	+	17 OY b, deep chrome
0.65	+++	15 mm.	+	—	+	17 OY d, light orange yellow
0.80	+++	17 mm.	—	—	+	17 OY f, pale orange yellow
0.65	++	15 mm.	—	—	+	15 YO f, pale yellow orange
0.65	+	Slight	—	—	+	15 YO d, orange buff
0.80	+	18 mm.	—	—	—	17 OY b, deep chrome
0.65	+++	13 mm.	+	—	+	17 OY f, pale orange yellow
1.70	+++	11 mm.	—	—	—	17 OY b, deep chrome
0.55	+++	16 mm.	—	—	+	19 YO y, light cadmium
0.70	++	15 mm.	—	—	+	17 OY b, deep chrome
0.80	—	15 mm.	—	—	+	15 YO b, capucine yellow
0.80	+	20 mm.	—	—	+	15 YO d, orange buff
0.90	—	15 mm.	—	—	+	15 YO d, orange buff
1.65	+	17 mm.	+	—	+	19′ YOY d, naples yellow
			GROUP E—*Continued*			
0.00	+	0 mm.	—	—	—	Scant growth
–0.05	—	3 mm.	—	—	+	21″ OYY f, ivory yellow
			GROUP F—*Continued*			
0.70	++	10 mm.	—	—	+	Slight pigment
0.95	—	0 mm.	—	—	—	21″ OYY f, ivory yellow
0.80	—	7 mm.	—	—	+	21″ OYY f, ivory yellow
0.90	+	3 mm.	—	—	+	21″ OYY f, ivory yellow
0.90	—	6 mm.	—	—	+	21″ OYY f, ivory yellow
0.80	+	Slight	—	—	+	21″ OYY f, ivory yellow
1.35	+++	5 mm.	—	—	+	21″ OYY f, ivory yellow
0.85	—	2 mm.	—	—	+	21″ OYY f, ivory yellow
0.85	+	13 mm.	—	—	+	19′ YOY f, cream color
1.05	+	9 mm.	—	—	+	21″ OYY f, ivory yellow
0.75	+	12 mm.	—	—	+	21″ OYY f, ivory yellow
1.25	+	14 mm.	—	—	+	21″ OYY f, ivory yellow
1.15	—	11 mm.	—	—	+	21″ OYY f, ivory yellow
1.05	—	11 mm.	+	—	+	19′ YOY f, cream color
1.10	—	11 mm.	—	—	+	19′ YOY f, cream color
1.15	+++	6 mm.	—	—	—	19′ YOY f, cream color
1.30	+++	5 mm.	—	—	—	19″ YY f, cartridge buff

TABLE 14 *Continued*

CHARACTERISTICS OF THE STAPHYLOCOCCI IN FRESHLY DRAWN MILK

Culture	Dairy	Cow	Action in Milk	Dex-trose	Lac-tose	Mal-tose	Raffi-nose	Man-nite
			GROUP F—*Continued*					
kx	2	14721	Curdled in 6 days..............	4.25	3.10	5.40	0.15	0.25
ky	2	73	Curdled in 6 days..............	3.00	3.40	5.40	0.15	−0.20
la	2	8	Curdled in 13 days............	2.70	3.15	3.40	0.05	−0.15
ld	2	5	Curdled in 6 days.......... ..	1.25	3.45	4.00	0.15	−0.10
lf	2	24125	Curdled in 4 days..............	4.35	3.50	3.30	0.20	−0.00
lj	2	10040	Acid, not curdled, in 14 days..	3.40	3.35	3.30	0.05	0.00
lk	2	20428	Curdled in 6 days..............	3.25	3.20	5.40	0.00	0.00
lo	2	11219	Acid, not curdled, in 14 days..	3.85	3.05	5.20	0.05	−0.10
lp	2	14776	Curdled in 6 days..............	4.20	3.30	4.95	0.15	−0.15
lq	2	16976	Curdled in 4 days..............	3.95	3.55	5.15	0.10	0.05
lu	2	10322	Curdled in 6 days..............	4.05	2.95	5.30	0.10	−0.10
lv	2	7563	Curdled in 4 days...	4.20	3.10	5.50	−0.20	0.00
ly	2	7563	Curdled in 6 days..............	4.45	3.90	2.95	0.25	−0.05
lz	3	454	Curdled in 2 days..............	3.65	2.95	4.90	0.05	0.05
ma	3	454	Curdled in 6 days..............	3.05	3.10	3.90	0.20	−0.10
mf	3	319	Curdled in 13 days............	3.95	2.90	4.20	0.25	−0.05
mo	3	278	Curdled in 13 days............	3.35	3.20	4.70	0.10	−0.20
mq	3	361	Curdled in 13 days............	3.45	2.80	4.45	0.25	0.30
my	4	335	Acid, not curdled, in 14 days..	3.15	2.60	5.80	0.35	0.10
nd	4	304	Curdled in 6 days..............	3.95	3.45	3.20	−0.05	−0.15
ng	4	315	Curdled in 12 days............	4.45	3.00	3.45	0.05	−0.10
ny	4	180	Curdled in 6 days..............	4.05	3.10	3.90	0.25	0.05
ob	4	62	Curdled in 4 days..............	4.40	3.25	4.20	0.10	0.15
oc	4	372	Curdled in 6 days..............	3.10	3.05	4.50	−0.05	−0.40
pa	5	12	Curdled in 7 days..............	1.15	3.40	2.90	−0.10	0.05
pe	5	18	Curdled in 7 days..............	4.00	3.80	2.80	0.15	0.20
pe	5	18	Curdled in 10 days............	1.20	3.50	3.00	0.00	0.10
pi	5	20	Curdled in 9 days..............	3.80	3.65	3.70	0.20	0.25
pl	5	21	Curdled in 7 days..............	4.95	3.15	2.70	0.00	0.10
pq	5	24	Curdled in 8 days..............	1.05	3.35	2.90	0.05	0.10
qb	5	31	Curdled in 7 days..............	4.55	3.05	2.60	−0.10	0.00
qd	5	31	Curdled in 7 days..............	1.00	3.20	2.85	0.10	0.10
qg	5	34	Curdled in 7 days..............	1.50	3.30	2.75	−0.25	0.15
qh	5	34	Curdled in 10 days............	4.55	3.85	2.80	−0.10	0.20
qp	5	36	Curdled in 8 days..............	1.20	3.60	2.55	−0.15	−0.10
ra	5	42	Curdled in 7 days..............	1.00	3.30	2.60	−0.05	0.20
rd	5	46	Curdled in 10 days............	4.70	4.00	2.45	−0.05	0.10
re	5	48	Curdled in 7 days..............	1.05	3.15	2.80	0.05	0.20
sa	5	59	Curdled in 11 days............	4.80	3.95	2.70	0.00	0.20
sj	5	73	Curdled in 9 days..............	1.40	3.10	2.60	0.05	0.10
			GROUP G					
bt	1	19	Curdled in 14 days............	3.70	4.30	3.25	0.15	0.10
bu	1	19	Curdled in 14 days............	3.90	3.95	2.55	0.00	0.10
bv	1	19	Curdled in 14 days............	4.15	4.00	3.10	0.30	0.25
ig	1	19	Curdled in 6 days..............	4.10	4.30	3.55	0.20	0.15
jz	1	8	Curdled in 11 days............	3.85	3.50	3.70	0.25	−0.05
kx	2	14721	Curdled in 6 days..............	4.25	3.10	5.40	0.15	0.25
lh	2	14758	Curdled in 6 days..............	2.85	2.65	2.60	0.25	0.15
ll	2	11814	Curdled in 13 days............	4.00	2.75	5.20	−0.05	0.30
lo	2	11219	Acid, not curdled, in 13 days..	3.85	3.05	5.20	0 05	−0.10
lw	2	20422	Curdled in 13 days............	3.95	3.65	4.90	0.15	−0.05
mb	3	454	Curdled in 5 days..............	3.80	3.15	2.10	0.20	0.05
md	3	060	Curdled in 6 days..............	3.65	2.90	4.90	0.30	0.00
mh	3	055	Acid, not curdled, in 14 days..	1.70	3.40	2.50	0.00	−0.10
mk	3	4	Curdled in 4 days..............	3.40	3.05	3.00	0.10	0.05
mr	3	349	Acid, not curdled, in 14 days..	3.55	3.15	1.45	0.25	0.05
ne	4	304	Acid, not curdled, in 14 days..	1.90	2.95	1.35	0.05	−0.10
oh	5	1	Acid, not curdled, in 14 days..	1.25	3.25	3.05	0.35	0.05
ph	5	20	Acid, not curdled, in 14 days..	3.65	3.65	2.20	−0.10	0.20
pm	5	21	Acid, not curdled, in 14 days..	4 20	3.60	1.40	0.05	0.15
pp	5	23	Curdled in 7 days..............	1.05	3.30	2.90	0.15	0.15
qo	5	36	Curdled in 7 days..............	1.20	2.25	3.50	0.00	0.25

TABLE 14—*Continued*
CHARACTERISTICS OF THE STAPHYLOCOCCI IN FRESHLY DRAWN MILK

Glyc- erin	Hemoly- sis	Lique- faction of Gelatin	Reduc- tion of Nitrates	Decom- position of As- paragin	Decom- position of Urea	Chromogensis

GROUP F—*Continued*

Glyc- erin	Hemoly- sis	Lique- faction of Gelatin	Reduc- tion of Nitrates	Decom- position of As- paragin	Decom- position of Urea	Chromogensis
0.85		16 mm.	—	—	+	19″ YOY f, cream color
1.05	+	10 mm.	—	—	+	19″ YOY d, cream buff
1.10	+	10 mm.	—	—	+	17 OY, cadmium yellow
0.95	—	12 mm.	—	—	+	19″ YOY d, cream buff
1.25	+ +	15 mm.	—	—	+	19′ YOY f, cream color
1.05	—	4 mm.	—	—	+	19′ YOY f, cream color
1.30	+ +	16 mm.		—	+	19′ YOY f, cream color
0.70	+	0 mm.	—	—	+	19′ YOY f, cream color
1.00	+	10 mm.	—	—	—	17″ OY d, pinkish buff
1.10	+	11 mm.	—	—	—	17 OY, cadmium yellow
1.15	+	11 mm.	—	—	+	19″ YOY f, cartridge buff
1.75	—	10 mm.	—	—	+	13 OY o, cadmium orange
1.95	—	13 mm.	—	—	+	19″ YOY d, cream buff
1.50	+	10 mm.	—	—	+	17 OY, cadmium yellow
1.25	+	10 mm.	—	—	—	19″ YOY f, cartridge buff
1.60	+	16 mm.	—	—	—	19″ YOY f, cartridge buff
1.10	+	13 mm.	—	—	+	19″ YOY d, cream buff
1.15	+	13 mm.	—	—	—	19″ YOY d, cream buff
0.80	+ +	0 mm.	—	—	—	23″ Yellow f, marguerite yellow
0.75	+	3 mm.	—	—	+	13 OYO, cadmium orange
1.15	+	11 mm.	—	—	+	21″ OYY f, ivory yellow
0.60	—	10 mm.	—	—	+	19″ YOY d, cream buff
2.10	+	25 mm.	—	—	+	13 OYO cadmium orange
2.05	+ +	11 mm.	—	—	+	19′ YOY f, cream color
1.20	+	17 mm.	—	—	—	19″ YOY f, cartridge buff
1.10	+ + +	15 mm.	—	—	—	15 YO, orange
2.30	+	15 mm.	—	—	—	21″ OYY f, ivory yellow
0.85	+ +	11 mm.	—	—	—	15 YO, orange
1.35	+	16 mm.	—	—	+	17 OY f, light buff
1.30	+ + +	15 mm.	—	—	+	17‴ OY d, vinaceous buff
1.75	+ + + +	14 mm.	—	—	+	19″ YOY f, cream color
1.15	+ + +	15 mm.	—	—	—	19″ YOY f, cartridge buff
1.50	+ + +	15 mm.	—	—	—	21″ OYY f, ivory yellow
2.20	+ + +	14 mm.	—	—	—	13 OYO, cadmium orange
2.45	+	15 mm.	—	—	+	13 OYO, cadmium orange
1.35	+	15 mm.	—	—	—	19″ YOY f, cartridge buff
3.00	+	15 mm.	—	—	+	15 YO, orange
1.35	—	13 mm.	—	—	—	21″ OYY f, ivory yellow
1.45	+	15 mm.	—	—	+	13 OYO, cadmium orange
2.50	+	14 mm.	—	—	+	13 OYO, cadmium orange

GROUP G—*Continued*

Glyc- erin	Hemoly- sis	Lique- faction of Gelatin	Reduc- tion of Nitrates	Decom- position of As- paragin	Decom- position of Urea	Chromogensis
−0.10	—	4 mm.	—	—	+	17 OY b, deep chrome
0.30	—	8 mm.	—	—	+	19′ YOY f, cream color
0.30	—	4 mm.	—	—	+	19′ YOY f, cream color
0.20	+	15 mm.	—	—	+	19′ YOY f, cream color
0.30	+	10 mm.	—	—	—	21″ OYY f, ivory yellow
0.50	—	16 mm.	—	—	+	19′ YOY f, cream color
0.30	—	20 mm.	+	—	—	19″ YOY b, chamois
0.00	—	0 mm.	—	—	+	19″ YOY d, cream buff
0.45	+	0 mm.	—	—	+	19′ YOY f, cream color
0.45	—	15 mm.	—	—	+	13 OY, cadmium orange
0.20	+ + +	15 mm.	—	—	+	23 Y f, Martius yellow
0.35	—	8 mm.	—	—	+	19′ YOY f, cream ,color
0.15	+ + +	0 mm.	—	—	+	19 YOY, light cadmium
0.25	+ + +	15 mm.	—	—	+	23′ Y, strontian yellow
0.30	+	25 mm.	—	—	+	15 YO, orange
−0.10	+	0 mm.	—	—	—	19 YOY b, apricot yellow
−0.05	—	3 mm.	—	—	+	21″ OYY f, ivory yellow
0.45	—	20 mm.	—	—	—	17 OY d, light orange yellow
0.10	—	0 mm.	—	—	—	17 OY, cadmium yellow
0.35	—	14 mm.	—	—	+	21″ OYY f, ivory yellow
0.35	+ + +	28 mm.	—	—	—	21 OYY d, pinard yellow

TABLE 14—*Continued*
CHARACTERISTICS OF THE STAPHYLOCOCCI IN FRESHLY DRAWN MILK

Culture	Dairy	Cow	Action in Milk	Dex-trose	Lac-tose	Mal-tose	Raffi-nose	Man-nite
			GROUP G—*Continued*					
qt	5	39	Acid, not curdled, in 14 days..	3.95	2.85	2.20	0.30	0.10
qu	5	39	Acid, not curdled, in 14 days..	3.95	3.90	2.70	−0.05	0.10
ro	5	54	Acid, not curdled, in 14 days..	4.20	1.10	2.15	0.00	0.20
sh	5	73	Curdled in 14 days.............	1.10	3.30	1.60	0.10	0.20
sk	5	73	Curdled in 4 days..............	4.70	3.15	2.95	0.25	0.00
			GROUP H					
as	1	14	Acid, not curdled, in 14 days..	4.00	3.30	0.35	0.15	0.05
fh	1	25	Acid, not curdled, in 14 days..	5.00	3.95	0.20	0.05	−0.05
fy	1	24	Acid, not curdled, in 14 days..	2.45	2.45	0.50	0.25	0.45
ik	1	25	No change in 14 days..........	4.60	5.05	0.05	0.00	0.35
nw	5	195	No change in 14 days..........	2.90	3.05	0.00	0.15	0.30

In 32, or 16.1% of the whole number of samples studied, bacteria were not multiplying in numbers worth considering.

Three types of bacteria were found commonly present in milk from all 5 dairies; they were streptococci, staphylococci, and bacilli.

The ordinary milk-souring organism, Streptococcus lacticus (Kruse), was not found in any of the samples of milk. It does not appear to localize and multiply in the udder.

Long-chained streptococci which failed to give the reduction of litmus in milk cultures characteristic for Streptococcus lacticus were isolated from 29, or 15.1% of the whole number. The highest number found per cubic centimeter was 264,000.

Micrococci were found in 113, or 58.8% of the whole number. The highest number found per cubic centimeter was 80,000.

The majority of the micrococci were shown to belong to one group, which agrees in characteristics with the pyogenic staphylococci. The majority of cultures of this type were non-virulent; some cultures were virulent; 2 cultures possessed such a high degree of virulence that inoculated rabbits died in about 16 hours.

Three other types of micrococci from the udder gave reactions which differed from those of the pyogenic staphylococci sufficiently to separate them into distinct groups. One of these groups is described, but no name is suggested for it because of the small number of cultures studied. Another group was identified with M. luteus according to

TABLE 14—*Continued*
CHARACTERISTICS OF THE STAPHYLOCOCCI IN FRESHLY DRAWN MILK

Glyc-erin	Hemoly-sis	Lique-faction of Gelatin	Reduc-tion of Nitrates	Decom-position of As-paragin	Decom-position of Urea	Chromogensis
			GROUP G—*Continued*			
0.30	—	6 mm.	+	—	—	19′ YOY f, cream color
0.30	+ + +	14 mm.	--	—	+	13 OYO, cadmium orange
0.10	—	26 mm.	—	—	—	15 YO d, orange buff
0.45	+ + +	24 mm.	--	—	+	21 OYY d, pinard yellow
0.15	—	....	--	—	—	Slight pigment
			GROUP H—*Continued*			
0.40	+	0 mm.	—	—	--	21″ OYY f, ivory yellow
0.45	--	12 mm.	+	—	+	23″ Y f, marguerite yellow
0.00	+	0 mm.	—	—	--	Slight pigment
0.05	- -	10 mm.	+	—	—	21″ OYY f, ivory yellow
0.25	+	17 mm.	—	—	--	21″ OYY f, ivory yellow

the Winslow's classification. The third group was characterized by the rapid and complete peptonization of milk. The name Micrococcus caseolyticus is suggested for this group.

Peculiar strains of the types of bacteria commonly present in freshly drawn milk were sometimes found localized in the udders of several cows of one dairy. A few cases were found of peculiar species, unlike any of the other udder organisms, being localized in the udders of several cows of one dairy.

The bacilli commonly present in milk from all 5 dairies were shown to be related to B. abortus. Three varieties of this type were distinguished.

The variety of B. abortus occurring most frequently in the samples of milk is designated B. abortus var. lipolyticus because it decomposes butter fat. In its cultural characteristics this variety agrees closely with Bang's original description of B. abortus. Cultures of this variety were shown to be capable of imparting undesirable flavors and odors to cream kept under conditions to which cream is frequently subjected.

Two other varieties of B. abortus type differed considerably from the lipolyticus variety, but resembled the cultures isolated from pathogenic sources and studied for comparison.

Cultures of B. abortus type were isolated from 45, or 23.4% of the 192 samples studied. The highest number of these bacilli found per cubic centimeter was 50,000.

BIBLIOGRAPHY

1. Andrewes and Gordon: 35th Ann. Rep. Local Govt. Bd. [Gt. Brit.] 1905-06. Sup. Rep. Med. Off., p. 543.

2. Baehr: Arch. f. Hyg., 1910, 72, p. 91.

3. Bang: Ztschr. Thiermedicin, 1897. 1, p. 241.

4. Bruck and Hidaka: Arch. f. Dermat. u. Syph., 1910, 100, p. 165. Abstracted in Centralbl. f. Bakteriol., R., 1910-1911, 48, p. 321.

5. Conn, Esten, and Stocking: 18th Ann. Rep. Storrs Conn. Agr. Exper. Sta., 1906, p. 91.

6. Currie: Jour. Agr. Research, 1914, 2, p. 429.

7. Dreyer: Centralbl. f. Bakteriol., I. O., 1912, 67, p. 106.

8. Dumas: Ann. de l'Inst. Pasteur, 1914, 28, p. 213.

9. Evans: Jour. Washington Acad. of Sciences, 1915, 5, p. 122.

10. Evans, Hastings, and Hart: Jour. Agr. Research, 1914, 2, p. 167.

11. Flügge: Ztschr. f. ärztl. Fortbild., 1910, 7, p. 513. Abstract in Centralbl. f. Bakteriol., R., 1910-1911, 48, p. 737.

12. De Freudenreich: Rev. Gen. Lait., 1903, 2, pp. 361, 385, 409.

13. De Freudenreich: Rev. Gen. Lait., 1904, 3, pp. 416, 440, 462, 492.

14. Gorini: Atti della Reale Accademia dei Lincei, 1902, 11, p. 159.

15. Gorini: Rev. Gen. Lait., 1904, 3, p. 505.

16. Gorini: Rev. Gen. Lait., 1910, 8, p. 337.

17. Gorini: La Clinica Vet., 1914, 38, p. 707.

18. Harding and Wilson: Tech. Bull., N. Y. (Geneva) Agr. Exper. Sta., No. 27, 1913.

19. Harrison and Savage: Rev. Gen. Lait., 1912, 9, p. 121.

20. Hart, Hastings, Flint, and Evans: Jour. Agr. Research, 1914, 2, p. 193.

21. Hastings and Hoffmann: Research Bull. Wis. Agr. Exper. Sta., 1909, 6, p. 189.

22. McFarland: A textbook upon the pathogenic bacteria and protozoa, 1912, p. 75.

23. Moore: Proc. Soc. for Promotion of Agr. Science, 1899, p. 110.

24. Moore and Ward: Bull. N. Y. (Cornell) Agr. Exper. Sta., No. 158, 1899.

25. Nicolle and Césari: Ann. de l'Inst. Pasteur, 1914, 28, p. 219.

26. Nowak: Ann. de l'Inst. Pasteur, 1908, 22, p. 541.

27. Petruschky and Kriebel: Die Ursachen der Sommersterblichkeit der Säuglinge und die Möglichkeit ihrer Verhütung, 1904.

28. Rogers and Dahlberg: Jour. Agr. Research, 1914, 1, p. 491.

29. Rogers, Clark, and Evans: Jour. Infec. Dis., 1915, 17, p. 140.

30. Schroeder and Cotton: 28th Ann. Rep. U. S. Dept. Agr., Bur. An. Ind., 1911, p. 139.

31. Schulz: Arch. f. Hyg., 1892, 14, p. 260.

32. Sherman and Hastings: Creamery and Milk Plant Monthly, 1915, 3, p. 11.

33. Ward: Bull. N. Y. (Cornell) Agr. Exper. Sta., No. 178, 1900.

34. Winslow and Rogers: The systematic relationships of the Coccaceæ, 1908.

THE ETIOLOGY AND EXPERIMENTAL PRODUCTION OF HERPES ZOSTER*

WITH PLATES 8 TO 19

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In our preliminary note[1] we reported the production of herpes of the skin, tongue, and lips, and of lesions in the corresponding ganglia, in a large number of animals injected with streptococci isolated from the tonsils, the pyorrheal pockets, the sputum, and the spinal fluid in herpes in man. In this paper, we wish to review briefly the development of our knowledge of herpes, record the results of the cultures and other findings in the cases studied, give details of representative experiments, and discuss the significance of our results.

HISTORICAL REVIEW

The first reliable report of observations concerning the etiology of herpes zoster was that of von Bärensprung[2] in 1861. He advanced the theory of a nervous origin, and later[3] demonstrated an acute inflammatory condition of the ganglion corresponding to the region affected. C. Boeck, in 1878, demonstrated pus-infiltration of the gasserian ganglion in the case of a girl who died with meningeal symptoms and in whom herpes of the face had developed. Wyss[4] and Sattler[5] each reported a case of zoster of the ophthalmic branch of the trigeminal nerve. In the case reported by Wyss, in which death occurred 7 days after the appearance of the eruption, hemorrhages were noted in and around the gasserian ganglion; there was interstitial "purulent inflammation" of the ganglion together with small abscesses of the eye muscles. In Sattler's case, with death 14 days after the eruption, there were round-cell infiltration of the ganglion, destruction of the ganglion cells, and degeneration of the ophthalmic nerve, the other two branches of the trigeminal nerve being normal. Lesser[6] reported 3 cases of zoster of the trunk in which degenerative changes and hemorrhages were within and surrounding the ganglia corresponding to the area of herpes. In 1900 the classic work of Head and Campbell[7] appeared in which the etiology of herpes zoster was thoroughly considered on the basis of pathologic findings in 15 autopsies and 400 clinical cases which had come under Head's[8] observation during his notable work on the subject of referred pain in visceral diseases. They concluded that herpes zoster is a specific infec-

tious disease which confers immunity, the number of recurrences being between 1 and 2%; that the specific virus has an affinity for the nervous system, particularly for the ganglia, in which there are found inflammatory changes, acute or chronic, according to the length of time between the appearance and the eruption and death. They proposed to consider the disease as an acute posterior poliomyelitis, in contradistinction to acute anterior poliomyelitis. They were unable to demonstrate micro-organisms in the affected ganglia. There is much other evidence tending to prove the infectious nature of the disease. As early as 1892, Head described a form of herpes as an acute specific infection. Blaschko[9] corroborated many of the findings of Head and Campbell, and described several cases of herpes zoster with febrile disturbances and swelling of the regional lymphatic glands. Oppenheim[10] cited the case of a young man with a bilateral zoster in the ulnar region which was accompanied by severe swelling of the glands. He stated also that epidemics of the disease had been reported; notably, those cited by Oppenheim, by Rohi, Kaposi, Weis, Reily, and Dopter—in the latter instance a house epidemic. Sachs[11] reported an epidemic that occurred in Breslau.

Micro-organisms (diplococci) were demonstrated in the spinal fluid by Achard and Loeper, Widal, and Brissaud-Siccard (Oppenheim). Magnus[12] reported a case which is interesting in view of our experimental observations. The patient, a man 41 years of age, had had an attack of rheumatism 6 years before his last illness; otherwise he had been well. His last illness was marked by grave motor disturbances and 3 weeks before death there developed a herpes zoster over the left side of the chest. Of the 20 spinal ganglia examined, pathologic changes were found only in the 3rd dorsal; hemorrhages were visible macroscopically, while microscopically there was an engorgement of the blood vessels and these were surrounded by marked areas of round-cell infiltration, which were continued into the periphery of the ganglion. In the ganglion proper there were hemorrhages and areas where only remnants of the ganglion cells remained. The round-cell infiltration was especially marked in the periphery, about the blood vessels. It was not diffuse, but occurred in small circumscribed areas. A painstaking search failed to reveal any microorganisms in the ganglia. The findings in the cord were significant. In the 2nd and 8th cervical, and in the 1st, 2nd, 3rd, 5th, and 6th dorsal segments there were found circumscribed areas of round-cell infiltration, especially marked about the arteria centralis and its anterior-horn ramifications. In the 2nd dorsal segment in the center of the anterior horn within an area of round-cell infiltration adjacent to an engorged blood vessel, diplococci were demonstrated. Finally, Sunde[13] reported a case of ophthalmic herpes in which he found diplococci in the hemorrhagic gasserian ganglion.

Howard[14] showed that the reaction of the skin in simple herpes does not differ essentially from that in zoster, and that simple herpes of the skin about the nose and the lips, like herpes of the trunk in pneumonia and meningitis, is associated with lesions of the Gasserian and the spinal ganglia.

Trevisanillo[15] isolated pneumococci from the vesicles in herpes of the lips in 4 cases of pneumonia, and reproduced vesicular lesions by inoculating the organism in normal areas of skin in the same individual. The virulence of

[9] Arch. f. mikros. Anat., 1887, 30, p. 495. Arch. f. Dermat. u. Syph., 1894, 42, p. 295.
[10] Textbook of Nervous Diseases, 1911, 1, p. 574.
[11] Rev. prat. d. mal. cutan., 1907, 6, pp. 9, 49.
[12] Norsk. Mag. f. Laegevidensk., 1906, 4, p. 1429.
[13] Ibid., 1913, 11, p. 339.
[14] Amer. Jour. Med. Sc., 1903, 125, p. 256.
[15] Ann. dell' Inst. Maragliano, 1914, 7, p. 277.

the pneumococci was low, but could be increased with successive animal passages (white mice). Since pneumococci were recovered from the blood also, there is no reason to doubt the occurrence of lesions in the ganglia in these cases.

That herpes occurs in the mucous membranes of the viscera, the respiratory and digestive systems, and the kidneys has been the belief of some close clinical observers. Fernet[16] cited several cases in which the relation between visceral disturbances and a concomitant herpes zoster was very striking, this being particularly true of pharyngitis of a certain type ("angine herpetique") and fibrinous pneumonia. Fernet believed that the former was due to an inflammation of the glossopharyngeal nerve and the latter to lesions along the vagus and the sympathetic, affecting the peripheral nerve-endings which ramify in one or more lobes of the lungs. He called attention to the fact that herpes labialis, so commonly found in these diseases, occurs invariably on the same side as that on which the pneumonia or angina is found. As evidence of the occurrence of herpes in the digestive tract, he cited among other cases the following: A 5-year-old boy was seized in the evening with high fever, accompanied by severe chills, which continued throughout the night. In the morning the temperature was lower, but a marked angina was present, which subsided the following day. Two days later there was a similar attack of fever, this time followed by a marked herpes of the face, the neck, the trunk, and the extremities. The second day of this attack the patient had colic, with a profuse discharge of mucus and slightly blood-tinged stools. These attacks lasted 3 days and then the symptoms disappeared, together with the herpes of the skin. The evidence seems strongly in favor of a herpes of the lower bowel.

Bittorf,[17] Kanera,[18] Rosenberg,[19] and Rosenbaum[20] reported cases of renal colic appearing simultaneously with a herpes zoster corresponding to the affected kidney. Vetleson[21] cited a case in which there was apparently a herpes of the lower intestinal tract, the patient, on the second day after the eruption of an inguino-femoral zoster, complaining of a similar pain associated with colic in the lower abdomen. In this case cultures were made, and from the herpetic vesicles and the spinal fluid a gram-positive non-encapsulated diplococcus was isolated. Vetleson cited another interesting case in which pneumonia, meningitis, appendicitis, and herpes facialis occurred concomitantly. He considered this an illustration of a spread of the infection along the nerve trunks upward through the cervical ganglion to the meninges, and downward along the vagus and the sympathetic to the lungs and the appendix.

Hunt[22] suggested that the attacks of vomiting and slow irregular pulse in a case of herpes of the ear were probably due to involvement of the vagus. However, postmortem records in which lesions of the visceral nerves of the ganglia have been demonstrated, cannot be found.

The finding of diplococci in the blister fluid, in the spinal fluid, and in the ganglia in these isolated instances suggests strongly that herpes zoster is a streptococcus infection. The necessary experimental proof, however, has heretofore not been brought forward.

[16] Semaine méd., 1910, 30, p. 517.
[17] Deutsch. med. Wchnschr., 1911, 37, p. 290.
[18] Ibid., p. 638.
[19] Ibid., p. 788.
[20] Ibid., p. 1120.
[21] Tidsskr. f. d. norske Laegefor., 1913, 33, pp. 241, 289.
[22] Amer. Jour. Med. Sc., 1908, 136, p. 226.

Technic

The technic employed in the experimental production of herpes zoster was essentially that used by one of us (Rosenow[23]) in connection with experiments on appendicitis, erythema nodosum, and ulcer of the stomach. The cultures were made from pus expressed from crypts and abscesses in the tonsils; from the depths of pyorrheal pockets; and from blister fluid, obtained by means of sterile pipets. The spinal fluid was obtained by aspirating it gently into a sterile glass syringe so as to avoid possible contamination from the air. Inoculations were made on blood-agar plates and blood-agar slants (the former incubated aerobically, the latter anaerobically), and into tall columns (10 to 12 cm.) of 0.2% dextrose broth, to which sterile ascites fluid and sterile tissues were usually added. The cultures for injection were incubated usually from 18 to 48 hours at 37 C., the bacteria sedimented in the containers in which they were cultivated, the supernatant clear fluid decanted, and suspensions made in salt solution so that 1 c.c. of the emulsion contained the growth from 15 c.c. of broth. In all instances, at the time of injection control cultures were made of the suspensions on blood-agar plates to prove the viability of the organisms and to be used in further study of them. The injections were usually made intravenously, but intraperitoneal and subcutaneous injections were made in some instances; they included emulsions of extirpated tonsils, mixed cultures obtained from tonsils and pyorrheal pockets, pure cultures of streptococci from the spinal fluid in herpes zoster in man, and pure cultures of streptococci obtained from the spinal fluid or from the ganglia in animals showing experimental herpes. The primary cultures from the foci, usually mixtures of streptococci and staphylococci, were often injected directly and the bacteria studied later. The animals were handled carefully, examined daily, and often caged separately, to avoid accidental lesions of the skin which might be mistaken for herpes. Herpes about the mouth, the tongue, or the eyes was easily detected, while, because of the large amount of hair, herpes of the trunk was found during life only when the lesions were marked. The lesions of the trunk were best observed on the under surface of the skin after death. Chloroform was used to kill the animals that survived the injection (60%). The examinations were made as soon after death as possible.

In order to be sure that the streptococcus in herpes was not merely an invader of the ganglia secondary to some unknown filterable virus, injections were made of the filtrates of the streptococcus cultures. Tissues for microscopic study were fixed in formalin and Zenker's fluid. The sections were stained with hematoxylin and eosin and for bacteria by the Gram-Weigert method.

REVIEW OF CASES WITH RESULTS OF CULTURES AND OF ANIMAL EXPERIMENTS

The following is a review of the clinical facts in the cases studied, the results of the cultures and of the animal experiments, and the details of representative experiments.

Case 267

This was a case of recurring brachial herpes in a woman 45 years of age, otherwise in good health. The attacks had occurred usually in the spring and fall, following contraction of a cold but without symptoms of distinct tonsillitis.

[23] Jour. Amer. Med. Assn., 1914, 63, p. 903.

Feb. 10, 1915.—The tonsils were examined and found small, atrophic, and covered by the anterior pillars. Cultures were made from the pus expressed from pockets in the tonsils and, on February 13, practically a pure culture of a green-producing streptococcus on blood agar, short-chained in ascites dextrose broth, was obtained.

Four guinea-pigs, 3 rabbits, and 1 dog were injected; all developed herpes of the skin or of the mucous membrane of the tongue.

Rabbit 33.—Injected, Feb. 11, 1915, with the growth from 30 c.c. of ascites dextrose tissue broth.

Feb. 12.—Found dead. On removal of the skin several hemorrhagic vesicular areas were found over the left shoulder. A number of hemorrhagic vesicles were found at the juncture of the mucous membrane of the upper lip and the skin and adjacent to the lower incisor teeth. The tongue showed numerous vesicular areas, many of which were ruptured with the result that much of the mucous membrane of the tongue was absent (Fig. 16). The gasserian ganglia appeared edematous and on cross section showed small hemorrhagic areas. The 3rd and 4th left dorsal ganglia corresponding to the area of herpes over the shoulder were hemorrhagic, and there were a few small hemorrhages in the peritoneal coat of the appendix and sigmoid, 2 rather large edematous hemorrhagic areas of the mucous membrane of the stomach, and marked degeneration and acidity of the liver. There were no hemorrhages of the meninges, the brain, or the cord. The spinal fluid was slightly turbid and tinged with blood.

Feb. 13.—Blood-agar-plate cultures made from the blood and from the fluid aspirated from the hemorrhages in the skin yielded a large number of streptococci and a moderate number of staphylococci. Sections of the upper lip through the herpes area showed desquamation of epithelium and separation of cells, together with infiltration of the epidermis with red blood corpuscles and a few round cells (Fig. 17a). In the deeper layers of the hemorrhagic area (Fig. 17b) were a thrombosed blood vessel (Fig. 17c) and a rather large number of diplococci and chains, while near the surface staphylococci, also, were found. No bacteria were found in blood vessels that did not show changes, but in the thrombosed vessel a moderate number of streptococci were disclosed (Fig. 18). Also, portions of the sections that did not show changes were free from bacteria.

CASE 270

This was a typical acute thoracic herpes zoster in a woman 45 years of age. Without known cause the attack began, Feb. 9, with severe pain in the right side of the chest. The patient had once had frequent attacks of tonsillitis, but none since she had had acute rheumatic fever associated with jaundice 13 years before. For 3 months past she had lost in weight and had had a subacute arthritis of the right knee and symptoms suggestive of gastric ulcer.

Feb. 11.—There were marked redness and blistering over the painful area on the right side of the chest.

Feb. 12.—Her tonsils were removed.

Feb. 17.—She was free from pain and the herpetiform lesions were nearly healed. Cultures from the emulsified tonsils showed practically a pure growth of a slightly green-producing streptococcus.

The emulsion from the tonsils in NaCl solution and the cultures in ascites dextrose broth of streptococci as isolated, and after one animal passage, when

injected intravenously into 6 dogs and 10 rabbits, produced herpes in 4 dogs and 4 rabbits. After 2 passages the streptococci produced marked herpes of the tongue in 1 rabbit, while after 3 and 4 passages the streptococci failed to cause herpes in the 3 rabbits injected. Of the 4 guinea-pigs injected intraperitoneally, 1 developed herpes over the left shoulder and over the lumbar region. Three rabbits injected after the strain had been cultivated for 1 week failed to develop herpes.

Rabbit 37.—Injected, Feb. 13, with 15 c.c. of the emulsion of the tonsil in NaCl solution.

Feb. 16.—It seemed well. Chloroformed. A circumscribed vesicular eruption on the under surface of the skin of the lateral aspect of the head was found. The areas of herpes measured from 0.5 to 1 cm. in diameter, were bilaterally placed, brownish in color, and situated in the skin proper. On cross section of these areas, much fluid exuded. There was suppurative arthritis of the left shoulder-joint. There was a rather large amount of distinctly turbid blood-tinged cerebrospinal fluid.

Feb. 19.—Cultures from the blood and the right gasserian ganglion gave a pure growth of Streptococcus viridans; those from the shoulder-joint, Streptococcus viridans and hemolytic streptococci; the fluid from the vesicular areas in the skin was sterile.

Dog 217.—Feb. 13, a medium-sized white dog was injected intravenously with the growth from the original culture from the tonsil in 75 c.c. of dextrose tissue broth.

Feb. 15.—The dog seemed well, but tender over the left lower thorax. Chloroformed. On removal of the skin, a number of circumscribed areas were found showing a hemorrhagic vesicular eruption opposite the 8th and 9th ribs on the left side. On shaving the skin it was seen that only one of these had blistered (Fig. 6); the others showed only marked hyperemia on the surface of the skin. An area of localized meningitis was found in the cortex between the hemispheres. The 8th and 9th dorsal spinal ganglia on the left side did not show hemorrhages on the surface, but on cross section showed distinct areas of hemorrhage. The joint fluid was turbid.

Feb. 16.—Cultures from the blood and from the edematous fluid in the vesicles and in the joint were found sterile. Sections of the 8th dorsal ganglion showed marked congestion, irregular staining of the ganglion cells, hemorrhage, especially in the sheath, round-cell infiltration (Fig. 7a), and thrombosis of the accompanying artery (Fig. 7b). The 9th dorsal ganglion showed similar changes, including a thrombosis of its vein. Altogether, 14 gram-positive diplococci were found in these ganglia. Serial longitudinal sections of the 8th and 9th dorsal ganglia and of the accompanying nerve roots showed that the thrombosed vessels were the artery and the vein supplying them. Cross-sections of the cord at the level corresponding to the 8th dorsal vertebra showed areas of hemorrhage in the gray matter in the posterior columns. The nerve cells surrounding the hemorrhage stained irregularly, and under high power considerable blood pigment was found. The blood vessel accompanying the posterior root just at the exit of the nerve fibers from the cord and beneath the dura was almost completely plugged by polymorphonuclear leukocytes and other cells (Fig. 8). Prolonged search resulted in the finding of 4 diplococci: 1 free in the vessel, 2 within leukocytes, and 1 in the wall of the vessel on the side of the nerve. Sections at two slightly different levels showed hemorrhages in the posterior nerve root and a thrombosed blood vessel under the dura directly opposite the posterior root.

This was a severe unilateral herpes zoster involving the left thoracic region, of 4 days' duration, in a laborer 49 years of age. The blistering was marked, the pain intense. The patient thought he had had high fever at the onset of the disease, but with the appearance of the eruption it had disappeared. He had never had a similar attack. The man was poorly nourished, seemed ill, coughed and raised small amounts of sputum, but the lungs were sterile.

Feb. 22.—The tonsils were found cryptic and the teeth surrounded by marked gingivitis. Cultures were made from the pus expressed from the tonsils, from the material drawn into a pipet from the inflamed gums, and from sputum, spinal fluid, blister fluid, and blood. The spinal fluid was clear, but smears from the sediment showed a moderate number of mononuclear cells in which there were a few diplococci.

Feb. 23.—Cultures from the blood were sterile; the spinal fluid gave a pure culture of a short-chained streptococcus and a few colonies of streptococci in ascites dextrose agar. Cultures from the tonsils, the teeth, and the sputum revealed chiefly green-producing colonies of streptococci, Micrococcus catarrhalis, and staphylococci. Cultures from the clear blister fluid remained sterile; those from the bloody blister fluid gave a few chains of diplococci, a spore-forming bacillus, and staphylococci.

The original cultures from the tonsils, from pus pockets about the teeth, and from sputum in ascites dextrose broth were injected intraperitoneally into 1 guinea-pig each, all 3 developing herpes. Intravenous injections of cultures of the streptococcus from each of these into 1 dog, 1 rabbit, and 1 guinea-pig each, were followed by herpes in all but 1 dog and 1 rabbit. The pure culture of the streptococcus from the spinal fluid of these animals caused herpes in 2 rabbits and 1 dog. After cultivation from 1 to 2 weeks, this streptococcus, when injected into 5 rabbits and 2 dogs, failed to produce herpes. The streptococcus from the spinal fluid (second culture) produced herpes of the eye and the lip and hemorrhage in the corresponding ganglion in a dog.

Rabbit 46.—Feb. 23, injected intravenously with the growth of a pure culture of Streptococcus viridans from the infected gums in 60 c.c. of ascites dextrose tissue broth.

Feb. 24.—Chloroformed. Marked herpes of the tongue (Fig. 13), marked areas of localized edema in the lung and the mucous membrane of the trachea suggestive of herpes were found. There were a moderate number of localized hemorrhages and edema of the mucous membrane of the stomach with 1 ulcer near the lesser curvature and 1 in the fundus. There was a moderate turbidity of the joint fluid from both knees. The spinal fluid was slightly blood-tinged. The gasserian ganglia appeared edematous, but no distinct hemorrhages could be made out.

Feb. 26.—The blood, the spinal fluid, the gasserian ganglia and the joint fluid yielded a large number of green-producing streptococci, and plate cultures from the ulcer in the cardiac end of the stomach gave 500 colonies of streptococci. Sections of the ganglia of the vagus nerve showed 1 rather large hemorrhage between the cell groups, and Gram-Weigert stains showed a few diplococci in the hemorrhagic area. Sections through the ulcerated area at the tip of the tongue showed desquamation, infiltration, and necrosis of the epithelium (Fig. 14b), slight round-cell infiltration of the underlying

muscle (Fig. 14a), and an aggregation of leukocytes in a small adjacent blood vessel (Fig. 14c). Stains for bacteria revealed a large number of gram-staining diplococci covering the ulcerated area (Fig. 15).

Dog 222.—Medium-sized brown and white dog, injected, Feb. 23, intravenously with the growth from 90 c.c. of an ascites-dextrose-broth culture from the tonsil.

Feb. 25.—It seemed well. Chloroformed. Marked hemorrhage and edema were found in the very lowest portion of the esophagus and the first portion of the stomach. This did not have the appearance of the usual hemorrhages, and the blood was unquestionably diluted with serous fluid. A number of herpetiform lesions could be found under the pleura in the left lung on the upper surface of the lower lobe. The largest of these measured 0.5 by 4 cm. (Fig. 23a). The contents of these areas consisted of hemorrhagic edematous fluid. The sympathetic and the vagus ganglia on the right side were imbedded in an area of hemorrhage; the spinal fluid was distinctly turbid; no lesions could be found in the spinal ganglia. The liver showed marked congestion; the joint fluids were clear.

Feb. 26.—Cultures from the blood, the joint fluid, the spinal fluid, the bile, and from the edematous fluid from the herpetiform lesions in the lung were sterile. Sections of the spinal cord, the spinal, the sympathetic, and the vagus nerves, and of a number of spinal ganglia, corresponding to the segment of the lesion of the stomach, showed no changes. The vagus and the sympathetic ganglia showed no gross lesions within their substance, but the capsules and the surrounding areolar tissue were hemorrhagic. The accompanying blood vessel of the sympathetic ganglion showed a beginning thrombosis; the thrombus filled one-half the lumen of the vessel and consisted chiefly of polymorphonuclear leukocytes and large mononuclear cells. Gram-Weigert stains showed diplococci in the hemorrhagic area and a few within the thrombus. A section of the herpes-like area in the lung showed dilatation of capillaries, hemorrhage and edema into alveoli, but little leukocytic infiltration (Fig. 24).

Rabbit 47.—Injected intravenously, Feb. 23, with the growth from 25 c.c. of an ascites-dextrose-broth culture from the sputum.

Feb. 24.—Found dead. Numerous small punctate hemorrhages were found in the skin of both ears, some of these distinctly vesicular in character. A ruptured vesicle was found near the inner angle of the right eye and near the juncture of the cornea. The left eye was normal except for some hemorrhages and edema, especially over the lower lid. A number of these had ruptured and glued together the margins of the lids. On the upper left lid a distinct vesicle in the subcutaneous tissue was found. There were a few lesions in the fascia covering the subscapular muscles on the right side. The spinal cord and the ganglia showed no apparent change. The gasserian ganglia appeared edematous. The middle ears showed a marked exudation; and there was infiltration of pus along the auricular branches of the 5th nerve as they passed along the anterior upper aspect of the temporal bone. Fluid from the knee joints was turbid, smears revealing leukocytes and diplococci.

Feb. 26.—A large number of green-producing streptococci in pure culture were obtained from the blood, the spinal fluid, and the gasserian ganglia. Sections of the left gasserian ganglion showed small areas of hemorrhage and round-cell infiltration, which was most marked in an area at the juncture between the nerve and the ganglion. The infiltration extended along the nerve

sheath for a considerable distance (Fig. 9a). A rather large number of diplococci were found in the infiltrated areas, but not in the normal portion (Fig. 10). Sections of the spinal cord showed no changes, but the left spinal ganglion at a level corresponding to the lesions found in the shoulder showed slight leukocytic infiltration and small hemorrhages.

Pig 59.—A medium-sized white and black guinea-pig was injected intraperitoneally, Feb. 23, with the growth from 15 c.c. of an ascites-dextrose-broth culture from the sputum.

Feb. 24.—Very ill. Chloroformed. Herpes of the upper lip and inner nostrils was seen only from the under surface of the skin. There were serofibrinous peritonitis and pleuritis; marked hemorrhages of the stomach; marked degeneration and acidity of the liver. There were no gross lesions of the gasserian ganglia, but sections showed areas of hemorrhage, in which were found a number of diplococci. No diplococci could be found in the more normal portion.

Dog 221.—Medium-sized fox terrier, injected, Feb. 23, with the growth from 90 c.c. of an ascites-dextrose-tissue-broth culture from the sputum.

Feb. 25.—Lame in the left hind leg. Chloroformed. There was one herpetiform lesion in the subcutaneous fat over the left shoulder blade and the fluid from the left knee was turbid. There were no other gross findings except a large amount of the usual turbid spinal fluid.

Feb. 26.—Cultures from the joint fluids and from the blood and the spinal fluids were sterile. Sections of the cord showed no hemorrhages. The ganglia and the associated nerve roots showed areas of round-cell infiltration, in which a few diplococci were found.

Case 278

This was a typical severe thoracic herpes zoster in a robust laborer 44 years of age. The area involved showed numerous small and large blisters. It was supplied by the left 7th and 8th intercostal nerves and extended from the median line in the back to the median line in front. The pain, which had begun 3 days before, was still severe; blistering had occurred 1 day before. The patient had been well for years except for an attack of sciatica 9 months before. Cultures were made from the small amount of pus expressed from the chronically infected tonsils and from the pus aspirated from the depth of several pyorrheal pockets and from the blister fluid.

Feb. 23.—Cultures on blood-agar plates from the tonsils and pyorrheal pockets yielded chiefly Streptococcus viridans, a few colonies of hemolytic streptococci, and a moderate number of staphylococci. The cultures from the blister fluid were sterile. Smears from the cultures in ascites dextrose broth showed very long chains and clumps of streptococci, larger short-chained diplococci resembling pneumococci, and staphylococci.

March 12.—The patient was examined again and cultures made exactly as before. He had been entirely free from pain for 10 days and feeling as well as ever.

The cultures from the teeth, when injected into 1 rabbit, failed to produce herpes. The original culture from the tonsils, containing both staphylococci and streptococci, produced herpes in 2 out of 3 rabbits and in 1 dog. The mixed culture of streptococci and staphylococci obtained from the spinal fluid of the dog showing herpes, produced herpes in 2 dogs and 1

rabbit. The filtrate (proved to be sterile) from the broth culture from the tonsil, did not cause herpes in the 2 rabbits injected. The culture obtained from the tonsil on March 12 after recovery, injected into 3 rabbits, developed herpes in 1.

Dog 225.—A large brown and white dog, injected intravenously, Feb. 24, with the growth from 90 c.c. of an ascites-dextrose-broth culture from the tonsil.

Feb. 25.—It seemed ill and in pain. Slight pressure over the lower thorax caused the animal to yelp. There were no visible changes of the skin, and no herpes about the mouth or the eyes.

Feb. 26.—The dog seemed somewhat better than it had been the day before. No herpes could be made out, but the skin over the right lower thorax was distinctly hyperemic.

Feb. 27.—At 9 a. m., the dog was very sensitive over the thorax. There were 2 large bluish-red edematous areas, approximately 5 by 8 cm., in the skin over the lateral and lower aspects of the thorax. Slight pressure here caused the dog to yelp. The pain seemed to be especially severe at intervals of from 10 to 30 minutes; at such times it cried out and rolled from side to side as if in great pain. The animal was alert mentally and when petted or spoken to kindly, wagged its tail. The condition grew worse, breathing labored. A number of blisters appeared over the area described. The animal was then chloroformed and examined at once. The skin over the lower and anterior portion of the thorax on both sides showed marked hemorrhage, edema, swelling, and blistering over areas approximately 6 by 10 cm., the long axis being parallel with the ribs. These were surrounded by numerous smaller herpetic lesions (Fig. 11), some of which did not involve the thick hair-covered overlying epidermis. The edema and infiltration of the large areas extended through the wall of the chest over a small area (3 cm.) on both sides where the peritoneum was blistered. All gradations in the character of the fluid in the lesions, from a clear serous fluid to a deeply blood-tinged and bloody purulent fluid, were found. The larger areas appeared infected. It was impossible to shave away the hair over these areas without causing the blisters to rupture. Herpetiform lesions were also found over the lower portion of the abdomen and of the prepuce, and under the right shoulder blade. The peritoneal cavity contained a moderate amount of turbid fluid. The stomach, in extreme spasm, contained no food but a moderate amount of bloody mucus, which reacted faintly acid to litmus. The mucous membrane of the fundus and of the pyloric end of the stomach showed edematous raised and opaque areas, in which were found many small hemorrhages and a number of superficial erosions. These areas had the typical appearance of herpetiform lesions. The liver showed marked congestion and mottling. The wall of the gall-bladder contained 4 circumscribed edematous hemorrhagic areas, resembling herpes. The kidneys appeared normal except for several circumscribed opaque edematous areas resembling infarcts. The lungs appeared normal except that there were altogether 7 subpleural circumscribed elevated collections of bloody serum having the appearance of vesicles. The myocardium was opaque and showed a number of small infarcts (2 by 5 mm.). The left auricle contained 2 herpetiform lesions at the juncture of the auricular appendix and the main body of the auricle. One of these appeared to involve the sinu-auricular node. The spinal ganglia in the lower cervical and upper dorsal region especially on the right side were imbedded in dark clotted blood. The hemorrhagic areas here were often fused, giving the appearance of an acute

diffuse pachyleptomeningitis. Similar but smaller hemorrhages were found around the ganglia and the cord in the lumbar region. The vagus and the right sympathetic ganglia were surrounded by small hemorrhages and appeared edematous. The brain, vagus and sympathetic nerves, the thyroid, adrenals, pancreas, the mucous membrane of the mouth, eye, and esophagus, the spleen, testicles, and the extremities appeared normal.

Feb. 28.—Cultures made from the brain substance, from the herpetiform lesions in the gall-bladder and the lung, from the bile and the joint fluid were sterile. The cultures from an infarct in the myocardium, from the blood, and from two subcutaneous lesions showed Staphylococcus aureus only. The more marked lesions in the skin showed staphylococci, a large bacillus, and streptococci. The peritoneal and spinal fluids yielded staphylococci and streptococci. Ascites dextrose agar-plate cultures of the emulsified tissue of two of the herpes-like areas in the stomach gave 30 and 50 colonies of streptococci. A section of the involved skin showed edema, leukocytic infiltration, and hemorrhage, especially of the deeper layers. The squamous epithelium in portions was raised, desquamated, and in some areas sloughed away (Fig. 12). The deeper cuboidal cells were everywhere intact. Gram stains showed clumps of cocci in diplococci and short chains, and a few bacilli in areas showing leukocytic infiltration. In the intercostal and abdominal muscles from the involved area were marked interstitial infiltration and hemorrhage. The spinal ganglia and the posterior nerve roots corresponding to the areas of herpes of the thorax were surrounded by marked hemorrhages and leukocytic infiltration, which extended into the sheath. The associated blood vessels showed partial thrombosis. A moderate number of diplococci and a few round single and clumped cocci were found in the hemorrhagic area showing blood pigment. Longitudinal sections of the subcutaneous nerves running into the involved area disclosed no changes except infiltration of the sheath in the involved area. Proximal to this point there were no changes. The vagus ganglion was surrounded by hemorrhages and leukocytic infiltration, chiefly of the capsule of the ganglion and the nerve sheath for a short distance (Fig. 25). Diplococci were found in the hemorrhagic area. The sympathetic ganglion showed no changes except slight hemorrhage beneath its sheath. The vagus and the sympathetic nerves remote from the ganglia showed no changes. The areas resembling herpes under the pleura in sections showed extravasation of blood into the distended alveoli. The blood corpuscles in the alveoli were separated and did not fill them completely.

Case 281

This was a case of recurring herpes zoster involving the upper and outer aspects of the right thigh in a nervous woman 40 years of age. The attacks had occurred yearly during early spring, and usually were not associated with distinct tonsillitis or other apparent infection. Three years before, she had had a severe attack of bilateral thoracic herpes zoster, and since then had had more or less distress after meals suggesting duodenal ulcer. The attack here described began 10 days after the onset of a typical attack of diphtheria. Seven days after the temperature had become normal (2 days before the attacks described) and after the throat cultures had failed to show diphtheria bacilli, the patient began to have pain in the outer aspect of the thigh, followed by redness and blistering on the third day after the appearance of pain.

Feb. 25.—The tonsils were small but red; from the crypts was expressed a small amount of fluid pus. Cultures were made from this pus and from the blister fluid.

Feb. 26.—Blood-agar-plate cultures gave a large number of green colonies of streptococci and of Micrococcus catarrhalis. Loeffler's serum slants yielded no diphtheria bacilli. Smears from the ascites-dextrose-tissue-broth cultures showed pure culture of a short-chained streptococcus. The cultures from the blister fluid were negative.

March 3.—The patient had fully recovered, pain was absent, the blistered areas nearly healed. Cultures were again made from the tonsils.

March 4.—Blood-agar plates inoculated with material from the tonsils showed, as before, many green colonies of streptococci, a few colonies of hemolytic streptococci, and of Micrococcus catarrhalis, but smears from the broth cultures showed much longer chains of streptococci than those in the former culture.

The primary culture from the tonsil pus during the attack was injected into 2 dogs and 2 rabbits. Only 1 rabbit developed herpes. The culture of the streptococcus obtained pure from the spinal fluid of this rabbit was injected into 6 rabbits and 1 dog, of which 4 rabbits developed herpes, the result in the dog being negative. The filtrate of the broth culture, thought to be sterile, but from which a few streptococci were isolated later, produced herpes of the right lower abdomen and of the outer aspect of the right thigh, associated with hemorrhage of the corresponding ganglion in the one rabbit which was injected (Rabbit 56, Case 281), and herpes of the upper lip in 1 of 2 dogs previously injected with the growth from the tonsil. A portion of this filtrate was then refiltered and again injected intravenously into a rabbit of the same size; no herpes developed and cultures made from the sediment of a centrifugated portion remained sterile. The cultures made after the patient had recovered failed to produce herpes in the one rabbit injected.

Rabbit 62.—Injected intravenously, Feb. 26, 1915, with the growth from 45 c.c. of an ascites-dextrose-tissue-broth culture from the tonsil.

Feb. 28.—The rabbit seemed ill and in pain. There was a marked herpes of the skin of both ears. Chloroformed. The skin of both ears was studded with numerous small hemorrhages and vesicular areas (1 to 7 mm. in diameter). These bore no relation to the site of the injection. A number of the vesicular areas had ruptured, and the dried serum covered the area. On cross section of a number of these a relatively large amount of sero-sanguineous fluid exuded. There was herpes of the skin over the upper aspect of the thighs (more marked over the right) and over the lower abdomen (Fig. 2). The herpes followed the distribution of the cutaneous nerves. The mucous membrane of the tongue was edematous at points, and a few ruptured blisters were found along the margin. The duodenum showed 4 edematous hemorrhagic areas 1 cm. beyond the pyloric ring. Three of these were distinctly vesicular, while the fourth was a small submucous fading hemorrhage. The gall-bladder wall contained 6 small whitish edematous areas over the fundus. The kidneys were pale and presented a peculiar appearance (Fig. 20). The capsule was raised in areas by clear fluid, which escaped on section. When the capsule was stripped, much fluid exuded, and some of the edematous opaque areas were found to extend into the cortex for a considerable distance. The cut surface was very moist. The medulla was grayish-red. The mucous membrane of the pelvis was edematous and blistered in areas. The mucous membrane of the ureters and the bladder showed no changes. The pericardial sac contained a moderate amount of turbid fluid; the parietal layer was edematous. The myocardium was mottled grayish-red; the endocardium was normal. The lungs showed a peculiar mottled appearance, but no distinct vesicles

could be found. The right gasserian ganglion was edematous. The right auriculo-temporal nerve was surrounded by pus. The drum-membrane and the lining of the middle ear on this side were edematous and hemorrhagic, and the cavity contained a thin pus in which a number of diplococci were found. The left gasserian ganglion and the middle ear presented a similar appearance. The meninges were dry; the brain and the cervical and dorsal ganglia appeared normal. In the lumbar region extradural hemorrhages surrounded the posterior nerve roots and ganglia. No gross hemorrhages could be made out on the freshly cut surface of the ganglia and cord. The sympathetic ganglion on the right side and the vagus ganglion on the left side were surrounded by hemorrhages. Those on the opposite sides showed less change. Diplococci, showing disintegration, were found in the hemorrhagic area surrounding the left vagus ganglion (Fig. 22). Stomach, liver, pancreas, spleen, lymph glands, eyes, thyroid, and joints were normal.

March 1.—Cultures from spinal fluid, gasserian ganglion, pericardial fluid, kidney, and blood yielded short-chained green-producing streptococci only. Sections of the cord, of the nerve roots, and of the ganglia corresponding to the area of herpes showed slight round-cell infiltration and hemorrhages of the posterior horn, of the corresponding ganglia and the associated sheaths, and thrombosis of the accompanying vessels (Fig. 3). Diplococci here were easily found. Sections of a herpetiform lesion of the skin of the ear showed sloughing of the epidermis, round-cell and red-blood-cell infiltration and a few diplococci in the cutis vera.

Sections of kidney stained by hematoxylin and eosin exhibited circumscribed areas of edema. The parenchymatous cells of the tubules were swollen; the protoplasm was granular and vacuolated; the nuclei stained poorly, some retaining their form, others showing disintegration. Some of the glomeruli in these areas were distended, the cells separated and the spaces filled with a finely granular fluid. The adjacent areas appeared normal except for congestion of the blood vessels, small hemorrhages, and compression of the tubules and the glomeruli. These areas extended well through the cortex. The contrast between the edematous cells with poorly stained nuclei and the intermediate portion of compressed well-stained cells was striking. The medullary portion was normal except for marked congestion of the blood vessels (Fig. 21). Stains for bacteria showed many diplococci, which were found, in radiating lines, chiefly between and within the cells of the convoluted tubules of the areas showing the herpes, the more normal portions and glomeruli disclosing no bacteria.

Rabbit 56.—A large white Belgian hare injected intravenously, March 2 and 3, 1915, with 10 and 5 c.c. of the filtrate of the streptococcus culture in ascites dextrose tissue broth from the spinal fluid of Rabbit 62.

March 6.—It seemed well. Chloroformed. No gross lesions were found except several areas of herpes, 0.5 to 1 cm. in diameter, of the deeper layer of the skin over the left lower abdomen and over the upper and outer aspect of the right thigh. A thorough search for lesions of the corresponding spinal ganglia failed to show gross lesions, but they were saved for microscopic sections.

March 8.—Blood, spinal fluid, and lung were sterile. Sections of the spinal ganglia, the nerve roots, and the pia-arachnoid membrane showed distinct hemorrhages and round-cell infiltration. The associated larger vessels were surrounded by leukocytic infiltration. Gram-Weigert stains showed diplococci in the infiltrated areas.

Case 368

This was a case of lobar pneumonia with marked herpes of the lips, the nostrils, and of the left side of the face in a young man.

March 22.—The 5th day of the disease cultures were made from the tonsils and from the sputum.

March 23.—Cultures from the tonsils and from the washed sputum gave a large number of green-producing colonies resembling pneumococci. The former yielded also hemolytic streptococci and Micrococcus catarrhalis. The broth culture gave chains of diplococci.

The culture from the sputum in ascites dextrose broth was injected into 1 dog and 1 rabbit. The former developed herpes of the lip and the tongue; the latter of the left side of the face and head and a bronchopneumonia 48 hours after the injection. The culture from the tonsil was also injected into 1 dog and 1 rabbit. The former developed herpes of the lip; the latter, herpes of the conjunctiva of the left eye, and of the skin over the left side of the face and the thorax. The gasserian ganglion of the dog injected with the tonsil strain showed areas of hemorrhage (Fig. 19a) and round-cell infiltration (Fig. 19b). The cultures of the strain isolated from the spinal fluid of the rabbits injected with strains from sputum and tonsil failed to ferment inulin, but otherwise closely resembled pneumococci.

Case 382

This was an acute gangrenous thoracic herpes zoster in a man 56 years of age. Beginning March 30, 1915, the patient had pain in the right lower costal nerve for a week. The day before, a rash had appeared over the tender areas and during the night a number of small blisters had appeared. There was no other complaint, except nasal catarrh, for a year past. Leukocytes numbered 10,000; hemoglobin 90%. The tonsils were small, cryptic, and covered by the anterior pillars. By making pressure outside the left tonsil a small-sized abscess was ruptured. Cultures were made from the pus obtained.

April 2.—Cultures on blood-agar plates yielded chiefly Streptococcus viridans, a few hemolytic streptococci, and staphylococci.

Injection of the growth in an ascites-dextrose-broth culture from the tonsil into 2 rabbits, produced in one herpes of the left margin of the tongue, marked herpes of the conjunctiva of the right eye, moderate of the left, and herpes of the left side of the thorax; in the other, herpes of the lateral aspect of the abdomen. The pure culture of streptococci obtained from the spinal fluid of these rabbits produced herpes in 2 of 4 rabbits injected; in one of the left thorax; in the other, of the lip, the tongue, and the left side of the face.

During the night following the examination the symptoms became much worse, and on April 7, Dr. Kretchmer, who kindly referred the patient to us, reported that the patient was suffering excruciating pain and that there were numerous ulcers at the site of the lesions. It would seem that, during the examination or later, there had occurred a new invasion of the bacteria which were proved to have affinity for the posterior ganglia, converting a mild attack into a severe one.

Case 391

This case was one of acute herpes zoster in a man 43 years of age. Forty-eight hours after taking 5 grains of calomel, which was thought to have salivated him, the herpes began in the left lower lip, then spread to the left side of the tongue, to the left buccal surface, and thence to the left side of the

face, including the left ear. The pain was intense for several days before the eruption appeared, also throughout the attack and afterward—4 weeks in all—altho the herpes had healed. There were marked swelling and edema of the involved areas, especially of the ear, resembling erysipelas. This was thought to be a secondary infection due to the streptococcus, and antistreptococcus serum was given. A marked neurasthenic state developed after the herpes began. The man had had pyorrhea for years.

April 16.—The tonsils were found to be small, but hyperemic and visibly infected. There was pyorrhea, especially about the lower incisors. Cultures were made from the small amount of pus expressed from both tonsils and from that aspirated from the pyorrheal pockets.

April 20.—Blood-agar plates made from the cultures in ascites dextrose broth inoculated with the pus from the tonsils and from the teeth, showed Streptococcus viridans, Staphylococcus, and a few colonies of Bacillus influenzae.

The original culture in ascites dextrose broth from the pyorrheal pocket was injected intravenously into 2 rabbits and 1 dog. One rabbit and the dog developed herpes. In the rabbit, the herpes was situated under the eye and the left lateral thorax; in the dog, in the right upper lip and the tongue. The culture from the tonsil was also injected into 2 rabbits and 1 dog; of these, only 1 rabbit developed herpes, the skin over the right hip being involved. The relatively high incidence of herpes following these injections is in keeping with the fact that, while the acute symptoms had subsided, the pain was still present when the cultures were made.

Completely negative results were obtained following intravenous injection of the cultures from the tonsils in a case of recurrent herpes of the mouth, and with the cultures obtained from the nasal discharge in a patient suffering from acute rhinitis associated with simple herpes of the lip.

RESULTS OF THE CULTURES

Cultures were made in 11 cases of herpes. Of these 5 were marked thoracic herpes zoster; 1 was severe herpes of the left side of the face and the left ear; 3 were recurring (1 involving the left arm, 1 the outer and upper aspect of the right thigh, and 1 the mucous membrane of the mouth); 1 was a marked herpes of the lips and of the left side of the cheek during pneumonia; and 1, a mild herpes of the lip during an attack of acute rhinitis. In no instance was there acute tonsillitis. The pus pockets found in the tonsils were small. All but 4 patients had pyorrhea. The patients included 1 woman 45 years old, 9 men from 40 to 56 years old, and 1 young man. The cultures from the tonsils (all of which contained pus), from the sputum, and from the pyorrheal pockets yielded a preponderance of moist green-producing colonies of a non-encapsulated, gram-staining, short-chained, often lanceolate, streptococcus. The cultures from the clear blister fluid in human herpes were usually sterile, or showed a few colonies of a large stapylococcus; but in one patient (Case 276) cultures from a hemorrhagic blister fluid showed a few colonies of a green-producing streptococcus, of a large gram-staining spore-forming bacillus, and of staphylococci.

Cultures from the spinal fluid and the blood were made in only one case. The former gave a pure culture of the streptococcus; the latter remained sterile. The fermentative powers of 7 of these strains that were proved to have affinity for the ganglia were tested on various sugars in broth. All but 1 produced acid in saccharose; all but 2 in salicin; 3 fermented raffinose, and 2 mannite; none fermented inulin. In short, these streptococci had the features of a pneumococcus, except high virulence, capsule, and inulin-fermentative powers (Fig. 26). At times there were found in the tonsils and pyorrheal pockets a few hemolyzing colonies of streptococci and frequently small dry slightly green-producing colonies of a smaller streptococcus. This was true both in blood-agar plates made directly and in those from the ascites-dextrose-broth cultures. Micrococcus catarrhalis was commonly present in the usual numbers. The cultures differed from those from the tonsils in other diseases in that they showed an unusually large number of staphylococci. Anaerobic cultures on blood agar were not characteristic.

RESULTS OF INTRAVENOUS INJECTIONS

In Table 1 is given a summary of the results of the injection of the strains (1) when first isolated, (2) after cultivation for a time, and (3) after animal passage. Sixty-one animals were injected with 11 strains as isolated. Of these, 70% developed herpes of the skin (Figs. 1, 2, 6, and 11) ; 15% herpes of the eyelids—a total average of experimental herpes in 75%. The lesions varied from those very small, just recognizable, to others very large and marked (Dog 225).

TABLE 1

Localization of Streptococci from Herpes Zoster

Time of Injection of Streptococci	Strains	Animals Injected	Percentage of Animals Showing Lesions in				
			Appendix	Stomach	Duodenum	Gallbladder	Pancreas
				Hemorrhage	Ulcer		
When isolated..............	11	61	10	29	8	16	2
Later.....................	6	15	0	13	7	7	13
After animal passage.......	4	7	0	28	10	0	0

In some instances the tendency of the strains from the apparent atria of infection to produce herpes was so marked that it occurred in nearly all animals injected intravenously, and in some it developed even after intraperitoneal injection. Thus, in one instance (Case 276) the

original cultures in ascites dextrose broth from the spinal fluid produced herpes in guinea-pig, rabbit, and dog. The pure cultures of the streptococcus from the spinal fluid of these animals produced herpes in 2 rabbits and 1 dog. That the herpes is due to the streptococci injected and not to an ultramicroscopic organism is indicated by the fact that sterile filtrates of these cultures failed to produce the disease. Moreover, when the characteristic affinity was marked, only a few streptococci were necessary to produce the disease. Thus, in one rabbit herpes of the skin with lesions and streptococci in the corresponding spinal ganglion (Case 281, Rabbit 56) followed injection of what was thought to be a sterile filtrate, but which later by centrifugation and cultures from the sediment was proved to contain a few living streptococci (probably not more than 10). A portion of the filtrate was again filtered and injected. It was now proved to be free from streptococci for, accordingly, it failed to produce herpes. Injection of the emulsion of the extirpated tonsil in NaCl solution at the outset of an attack (Case 270, Rabbit 37), as well as of the original cultures in broth containing a mixture of bacteria, was followed by a herpes that was proved to be due to streptococci

The lesions were usually unilateral, especially after the injection of small doses, altho after the injection of large doses bilateral herpes occurred rather often. In some instances the location of the herpes in the animals corresponded rather closely to that in the patients from whom the cultures had been obtained (Case 281, Rabbits 62 and 56; Case 368, Dog 299).

TABLE 1—*Continued*

LOCALIZATION OF STREPTOCOCCI FROM HERPES ZOSTER

Percentage of Animals Showing Lesions in											
Intes-tines	Joints	Endo-car-dium	Peri-car-dium	Myo-car-dium	Mus-cles	Kid-neys	Lungs	Skin	Ton-gue	Eyes	Paro-tids
8	11	5	11	5	11	5	21	70	15	15	0
7	60	7	0	20	40	7	20	7	0	13	0
0	43	0	14	0	28	0	43	28	14	0	0

Table 1 shows further that after the streptococci are cultivated on artificial media (7 to 14 days) and after animal passage (2 to 5) they largely lose the power to produce herpes; it occurs in only 7 and 28% of the animals injected. Likewise, the cultures made from the pre-

sumable infection-atrium 7 to 10 days after recovery, produced herpes in only 1 of 9 rabbits and 3 dogs which had been injected. The patient (Case 391), however, who continued to have severe pain (post-herpetic neuralgia) 4 weeks after the lesions of the skin had healed, still harbored streptococci, which produced herpes in most of the animals injected.

The posterior roots or ganglia corresponding to the area of herpes of the skin (Figs. 1, 2, 6, and 11) nearly always showed hemorrhages and edema. Smaller hemorrhages were usually found about the neighboring ganglia in those animals with severe herpes, tho the corresponding area of the skin was free from herpes. The hemorrhage at times extended for a short distance along the sheath of the spinal nerve, but the intercostal nerves and the cutaneous branches remote from the lesions in the ganglia and the skin showed no gross or microscopic changes. The hemorrhages about the ganglia and the posterior root at times extended into the loose connective tissue of the external and posterior portion of the dura, presenting the picture of an external pachymeningitis. Herpes of the skin of the ears of rabbits, when present, was usually bilateral, and was nearly always accompanied by herpes of the tongue. In two rabbits with marked herpes of the external ears there were found what appeared to be herpes of the drum and middle ear and an infection extending from the gasserian ganglia along the auricular nerve. Herpes of the face, the eyelids, and the tongue was usually accompanied by hemorrhage of the gasserian ganglion.

Herpes of the viscera occurred chiefly after injection of large doses in animals showing marked bilateral herpes of the skin. In some instances, however, it occurred without herpes elsewhere (Case 276, Dog 222). Herpes has been observed of the lung and the pleura (Fig. 23), of the peritoneum, the gall-bladder, the mucous membrane of the stomach and duodenum, of the kidney (Fig. 20), and of the visceral pericardium. Herpes of the viscera was always accompanied by hemorrhage and edema about the ganglia of the vagus or the sympathetic nerve, or both. Lesions here were not found where visceral herpes was absent.

Cultures made in the case of animals after injection showed that the streptococci tended to disappear from the blood. Sixty percent of the animals survived the injection. This afforded opportunity to study the rôle played by the bacteria in the production of the disease. Routine cultures were made from the spinal fluid, the hemorrhagic area about

the ganglia, the blood, the blister fluid, and the joint fluid in the animals. The spinal fluid showed a characteristic turbidity, which was due chiefly to mononuclear cells. The cultures from the spinal fluid and from the hemorrhagic ganglia nearly always showed a larger or smaller number of colonies of the characteristic streptococcus, even when the blood and other cultures were sterile. In fact, this was common after injections of the primary cultures from the focus, containing in addition to streptococci, staphylococci. The streptococci from the spinal fluid in these animals showed a marked tendency to produce herpes when injected the second time, but in subsequent injections rarely produced herpes. Cultures from the herpetiform lesions in the skin, which showed no hemorrhage, and from animals that survived the injection, were usually sterile; while from the marked lesions showing hemorrhage and at times necrosis and beginning gangrene (Case 278, Dog 225), the organism injected, together with staphylococci and bacilli, was usually obtained. Localized meningitis was observed once. The occurrence of lesions in the other organs corresponds closely to the average incidence of lesions in these organs following the injection of streptococci from a wide range of sources.[23]

MICROSCOPIC ANATOMY OF THE LESIONS

Microscopic examinations of the milder herpetic areas in the skin showed separation of the cells, desquamation, and, usually, slight round-cell infiltration. Bacteria were not found in these. The marked lesions (Fig. 12) showed desquamation of the epithelium of the epidermis, hemorrhage, marked leukocytic infiltration, and at times thrombosed blood vessels (Fig. 17c). In these, staphylococci, streptococci, and bacilli were found. The sections in herpes of the tongue and the lip showed sloughing of the mucous membrane, partial or complete thrombosis of blood vessels, marked hemorrhage of the muscular layer, and leukocytic infiltration (Fig. 17). The blistered epithelium of the tongue sloughed promptly, and the ulcerated base revealed a large number of streptococci in all of the sections examined (Figs. 14 and 15). The lesions in the ganglia and in the adjacent nerve trunks consisted usually of small areas of hemorrhage and round-cell infiltration immediately surrounding the capsule of the ganglia or the associated nerve-sheath and around the accompanying blood vessels (Figs. 3, 5, 7, 8, 9, 19, and 25). The blood vessels of the ganglia and the posterior root

[23] Jour. Amer. Med. Asssn., 1915, .65, p. 1687.

usually showed partial or complete thrombosis (Figs. 3, 7, and 8), the thrombi consisting of polymorphonuclear leukocytes and large mononuclear cells and fibrin (Fig. 8).

Serial sections in one instance showed that the thrombus in the artery to the ganglion extended for a considerable distance along the posterior root and under the dura. Diplococci, often in large numbers, were found in the hemorrhagic and infiltrated areas of the spinal, vagus, and sympathetic ganglia, but not in the portions free from changes or in the normal ganglia (Figs. 5, 10, and 22). The bacteria have been found in the thrombosed blood vessels accompanying the lesions of the ganglia and posterior horns and in the peripheral lesions in the herpes of the lip and the tongue (Figs. 4 and 18). It must not be supposed that the finding of diplococci in the hemorrhagic infiltrated areas about the ganglia was a part of general invasion, because they were absent in the portions of sections showing no change and present, in many instances, in the lesions in animals seemingly well, with blood sterile. Longitudinal sections of the vagus, sympathetic, intercostal, and cutaneous nerves and of the accompanying sheaths remote from the lesions in the ganglia or skin showed no changes or bacteria. Sections of the spinal cord in a few instances showed hemorrhage and round-cell infiltration in the posterior columns.

SUMMARY AND GENERAL DISCUSSION

The streptococcus found by us, which has such marked affinity for the posterior root ganglia, resembles morphologically the diplococci found in the gasserian ganglia by Sunde.[13] The cells found in the spinal fluid in the animals injected are similar to the cells in the spinal fluid in herpes in man (Schottmüller).[24] The common occurrence in the spinal fluid of the streptococci in pure culture having affinity for the ganglia even when mixed cultures were injected is in accord with the finding of streptococci in the spinal fluid in one of our cases and in those of Achard and Loeper, Widal, and Brissaud-Siccard (cited by Oppenheim).[10]

The occurrence of relatively slight lesions in ganglia without peripheral herpes, adjoining those showing marked lesions accompanied by herpes; and the finding in the probable atrium of infection of bacteria having affinity for the posterior roots and ganglia in the patient who continued to have pain after the lesion of the skin had healed, and not

[24] Leitfaden zur Untersuchung der Zerebrospinalflüssigkeit, 1913.

in those patients who were free from pain, suggest strongly that the pain in post-herpetic neuralgia and allied conditions is due to active but slight infection of the ganglia or posterior roots. That these pains are not always due to scar tissue is certain, because Head and Campbell[7] have found that ganglia corresponding to the area of herpes zoster may be largely replaced by connective tissue without the patient's suffering pain. The occurrence of herpes zoster in patients who have ulcer of the stomach (Case 281) or empyema is probably not a reflex arc effect or infection of the ganglia by way of the nerve lymphatics, as believed by Orr and Rowe,[25] but is due to a hematogenous infection from a focus harboring streptococci that have an elective affinity for the structures involved. The occurrence in 8% of the animals of ulcer of the stomach (proved to be due to local streptococcal infection in the mucous membrane following injection of these strains), is in line with this idea.

The fact that visceral herpes with accompanying lesions of the vagus and sympathetic ganglia has been produced experimentally, supports the view held by clinical observers that visceral herpes occurs in man, and since it occurred chiefly in animals with severe, usually fatal, bilateral herpes, it affords experimental evidence in favor of the general impression that bilateral herpes is apt to end fatally.

The absence of streptococci in the clear blister fluid both in man and in animals when the organisms are present in the ganglia, and the presence of pain before peripheral lesions can be made out, suggest that the lesions in the ganglia are primary, and that the peripheral manifestations—herpes of the skin, the tongue, the lip, and the viscera—are secondary trophic effects. The occurrence of herpes in cases of pressure-paralysis of the spinal cord would seem to be in accord with this idea. The finding of the streptococcus in the turbid bloody blister-fluid in one case of our series in man and in the severer peripheral lesions—skin, mucous membrane, tongue, and viscera—in experimental disease, the common occurrence of thrombosed blood vessels containing diplococci in both the peripheral and central lesions, and the absence of bacteria and demonstrable changes in the intervening nerve trunks, suggest strongly that while the primary milder lesions are trophic, making a "locus minoris resistantiae," the severer lesions, presenting as they do all the features of an infectious process, are the result of a superimposed hematogenous infection.

[25] Brain, 1914, 36, p. 271.

It is a noteworthy fact that of the large number of animals injected with cultures from a variety of diseases other than herpes,[21] in which lesions resembling those in man have been produced, none has exhibited typical herpes.

Since the streptococci lose the characteristic affinity after cultivation on artificial media, after animal passage, and apparently in the focus of infection after recovery, the conclusion seems warranted that the atrium of infection is not only the place of entrance, but the place where the streptococci, by growth in symbiosis with other bacteria and under varying grades of oxygen-pressure, may acquire the peculiar properties necessary to infect in this particular manner.

It would appear, then, that herpes zoster is due to a streptococcus having elective affinity for the ganglia and the posterior roots. The possibility, however, that the disease in some instances may be due to other bacteria having a similar affinity must be admitted. (After this report was in proof, we found an abstract[26] of a paper by Raymond and Lot[27] which states that they cultivated a "cocco-bacillus" from the blood in 2 cases of herpes zoster, which when injected into rabbits and guineapigs, was followed by herpes and associated lesions of the corresponding ganglia. It is impossible to decide whether their organism is the same as ours, the original publication not being available.)

[26] Centralbl. f. Allgem. Path. u. Anat., 1915, 26, p. 501.
[27] Bull. et mem. d. soc. méd. d. Hôp. de Paris, 1913, 30.

EXPLANATION OF PLATES 8 TO 19

PLATE 8

FIG. 1. Herpes as seen on the under surface of the skin over the lower right thoracic region in a rabbit 24 hours after an intravenous injection of the streptococcus from herpes zoster (281). Note particularly the vesicular character of one of the lesions. Natural size.

FIG. 2. Herpes of the skin of the upper aspect of the right thigh of Rabbit 62, 48 hours after an intravenous injection of the streptococcus from the tonsil in Case 281. × 4.

PLATE 9

FIG. 3. Thrombosis of the vein (a) and perivascular infiltration (b) of the posterior root adjacent to the ganglion corresponding to the area of herpes shown in Fig. 2. × 110.

FIG. 4. Diplococci in a leukocyte within the thrombosed vein shown in Fig. 3. × 1200.

FIG. 5. Diplococci in the hemorrhagic and infiltrated area surrounding the spinal nerve in the vertebral foramen corresponding to the area of herpes shown in Fig. 2. × 1200.

PLATE 10

FIG. 6. Herpes of the skin over the left thorax of Dog 217, 48 hours after an intravenous injection of the streptococcus from the tonsil of Case 270. × 2½.

FIG. 7. Leukocytic infiltration in and surrounding a ganglion (a) and thrombosis of the adjacent artery of the spinal ganglion (b) corresponding to the area of herpes shown in Fig. 6. × 65.

PLATE 11

FIG. 8. Thrombosed blood vessel accompanying the posterior nerve root within the dura of the ganglion shown in Fig. 7. Note the large number of polymorphonuclear leukocytes. × 475.

PLATE 12

FIG. 9.—Hemorrhage and leukocytic infiltration (a) of a spinal ganglion and of the associated nerve in Rabbit 47, 24 hours after an intravenous injection of the streptococcus from sputum in a case of severe thoracic herpes zoster (276). × 60.

FIG. 10. Diplococci and streptococci in the infiltrated area shown in Fig. 9. × 1200.

PLATE 13

FIG. 11. A number of the smaller areas of herpes of the skin over the thorax of Dog 225, 72 hours after an intravenous injection of the streptococcus from the tonsil in a case of severe thoracic herpes zoster (278). Natural size.

FIG. 12. Section of the skin through the area of herpes (Fig. 11) over the thorax in Dog 225, 72 hours after an intravenous injection of the streptococcus from the tonsil in Case 278. Note the desquamation of the epidermis (a) and the leukocytic infiltration (b). × 35.

PLATE 14

FIG. 13. Marked herpes of the tongue in Rabbit 46, 24 hours after an intravenous injection of the streptococcus from a pyorrheal pocket in a case of severe thoracic herpes zoster (276). × 2¾.

FIG. 14. Section through a herpetic lesion at the tip of the tongue shown in Fig. 13. Note the hemorrhage and cellular infiltration (a), the ulceration (b), and the beginning thrombosis of an adjoining blood vessel (c). × 75.

FIG. 15. Diplococci covering the base of herpetic ulcer of the tongue shown in Figs. 13 and 14. × 1200.

PLATE 15

FIG. 16. Herpes of the tongue and of the mucous membrane about the teeth and lips in Rabbit 33, 24 hours after intravenous injection of the streptococcus from the tonsil in a case of recurring herpes (267). × 2¼.

FIG. 17. Section through a herpetic area of the lip of the rabbit shown in Fig. 16. Note the hemorrhage in the epidermis (a) and deeper layers of the skin (b), and the thrombosed blood vessel (c).

FIG. 18. Diplococci in the thrombosed blood vessel shown in Fig. 17 (c). × 1200.

PLATE 16

FIG. 19. Hemorrhage (a) and round-cell infiltration (b) of the gasserian ganglion in Dog 299, 48 hours after an intravenous injection of the streptococcus from the tonsil in a case of lobar pneumonia with marked herpes of the lip and cheek (368). × 60.

PLATE 17

FIG. 20. Herpes of the kidney of Rabbit 62, 48 hours after an intravenous injection of the streptococcus from the tonsil in Case 281. Note the numerous vesicles under the capsule. × 2¼.

FIG. 21. Section of the kidney shown in Fig. 19. Note the edematous areas (a), the compression of the glomeruli (b), and the swollen epithelium in the tubules with poorly staining nuclei (c).

FIG. 22. Diplococci in the hemorrhagic vagus ganglion of Rabbit 62, showing herpes of kidney shown in Fig. 20. × 1200.

PLATE 18

FIG. 23. Herpes (a) of the lung in Dog 222, 48 hours after an intravenous injection of the streptococcus from the tonsil of Case 276. × 1½.

FIG. 24. Section of the lung through the herpetic area shown in Fig. 23. Note the edematous fluid (a) and the hemorrhage (b) in the alveoli, and the absence of the pleura.

PLATE 19

FIG. 25. Hemorrhage (a) and leukocytic infiltration (b) in the sheath of the vagus just beyond the inferior vagus ganglion in Dog 225 showing visceral herpes 72 hours after an intravenous injection of the streptococcus from the tonsil in Case 278. × 110.

FIG. 26. Photomicrograph of a 24-hour culture in ascites dextrose broth of a streptococcus isolated from the spinal fluid in a rabbit showing herpes after intravenous injection of a streptococcus culture from the tonsil in a case of herpes zoster in man (281). The morphology is characteristic of other strains as well. Gram stain. × 1200.

PLATE 8
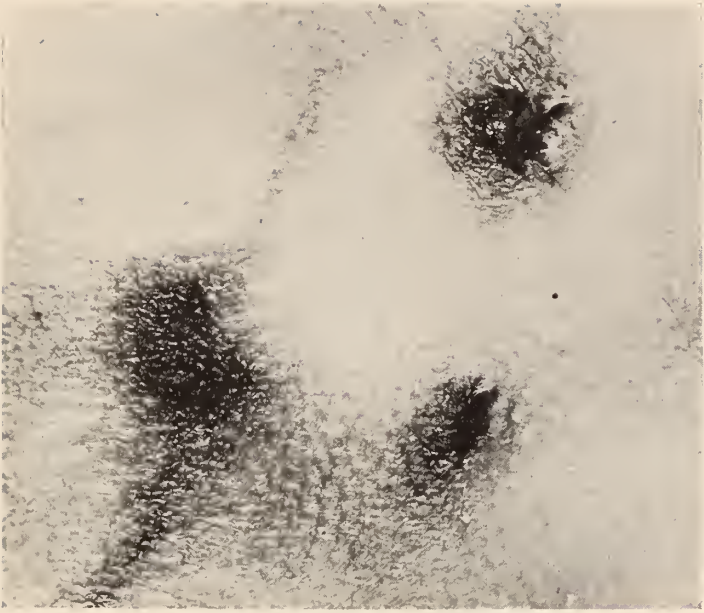
Fig. 1

Fig. 2

PLATE 9
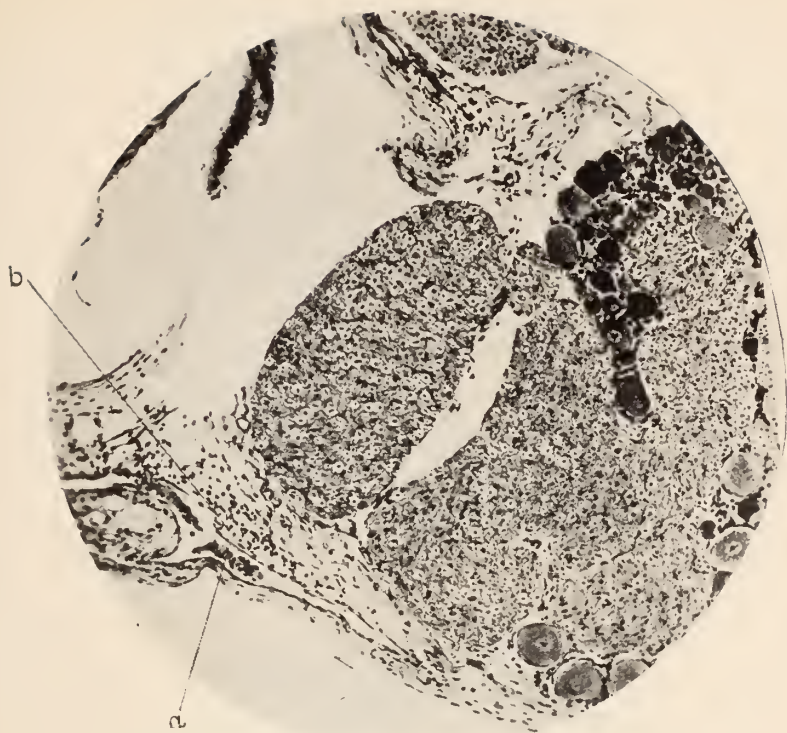
Fig. 3

Fig. 4
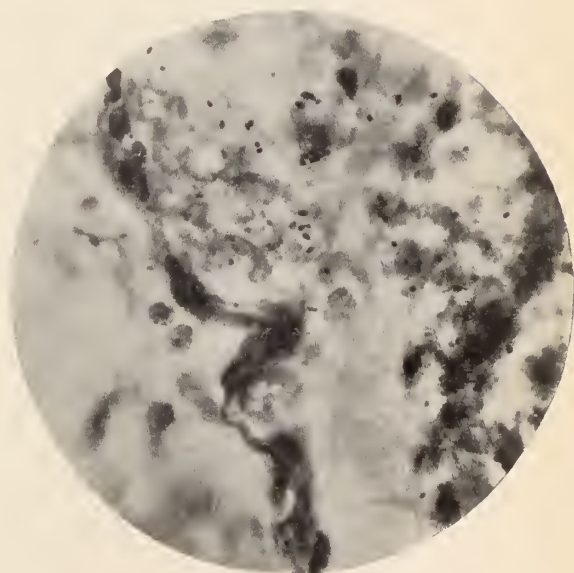
Fig. 5

PLATE 10
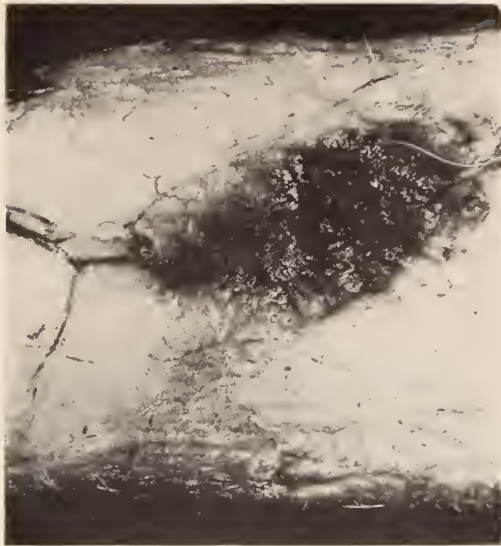
Fig. 6

Fig. 7

PLATE 11
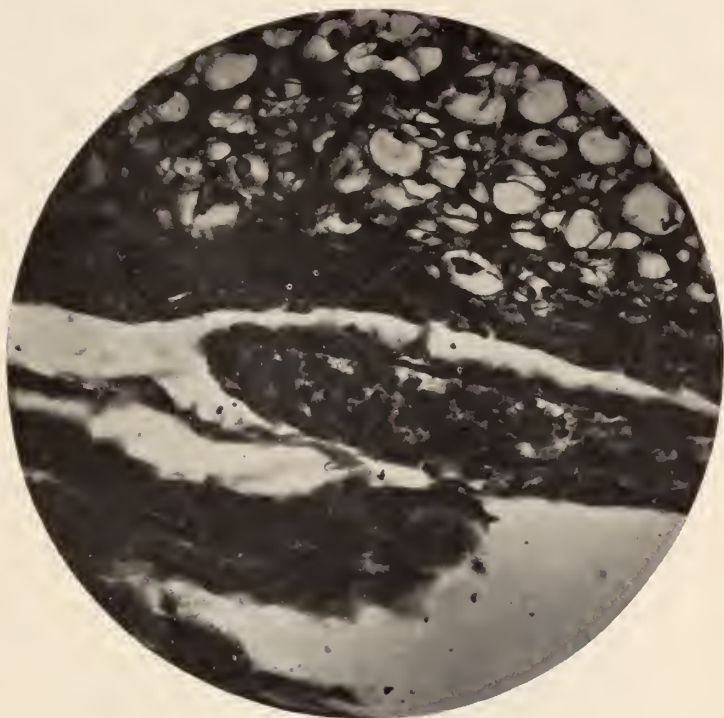
Fig. 8

PLATE 12
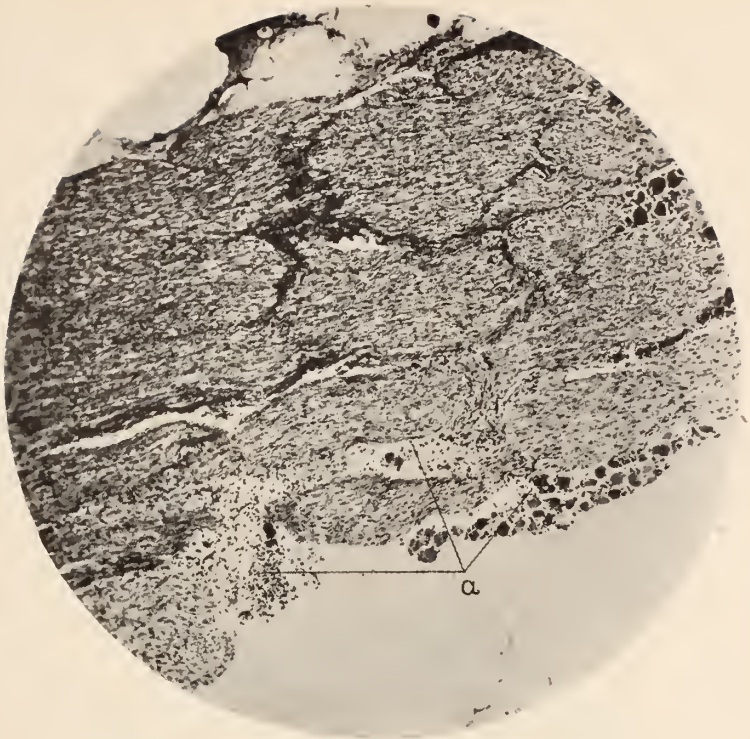
Fig. 9
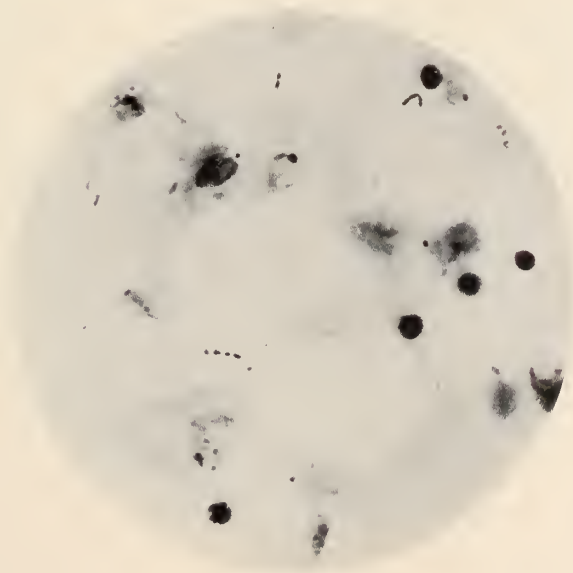
Fig. 10

PLATE 13
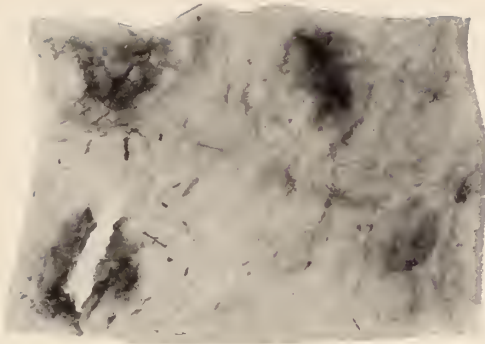
Fig. 11
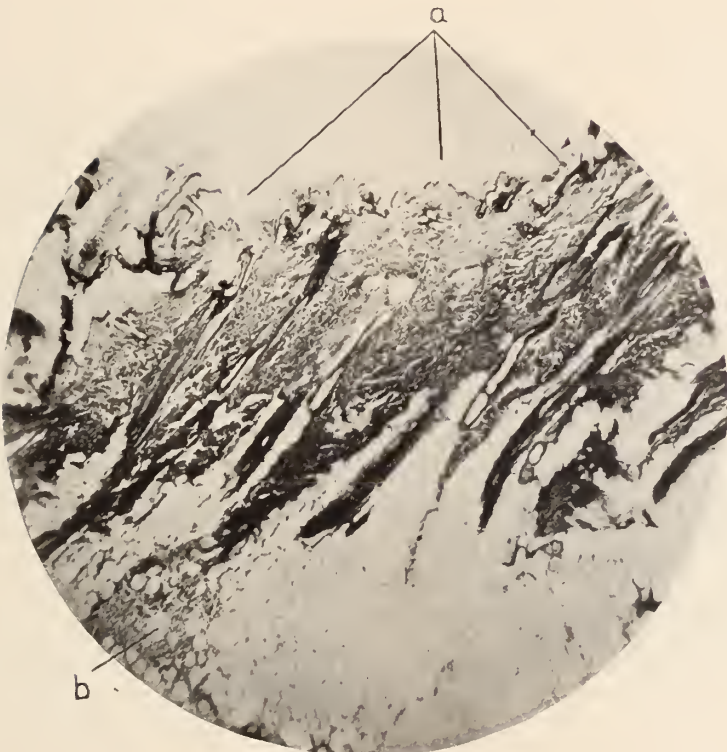
Fig. 12

PLATE 14
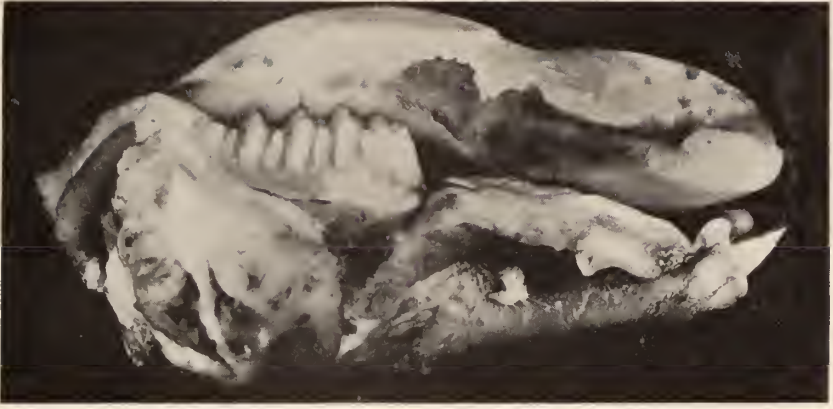
Fig. 13
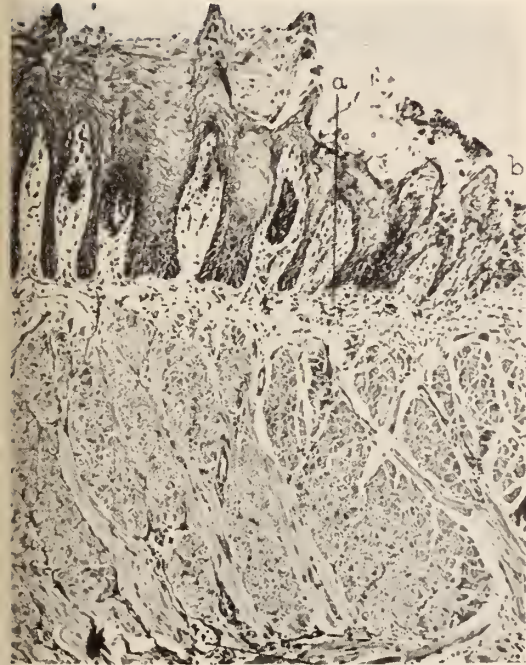
Fig. 15

Fig. 14

PLATE 15

Fig. 16
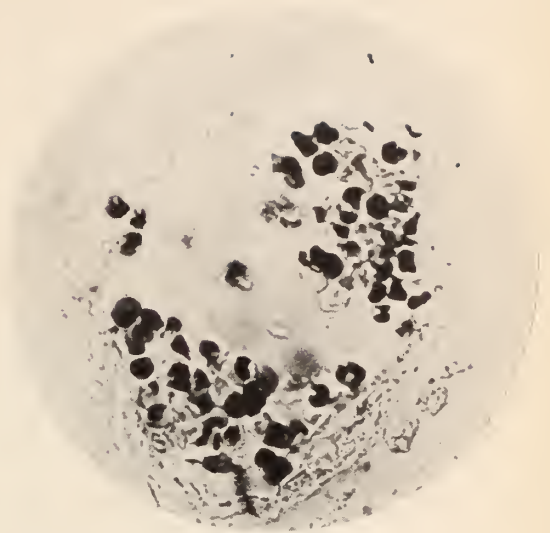
Fig. 18
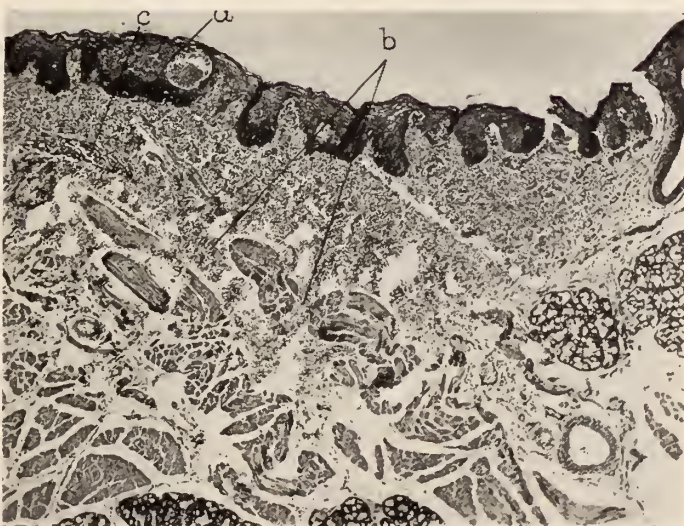
Fig. 17
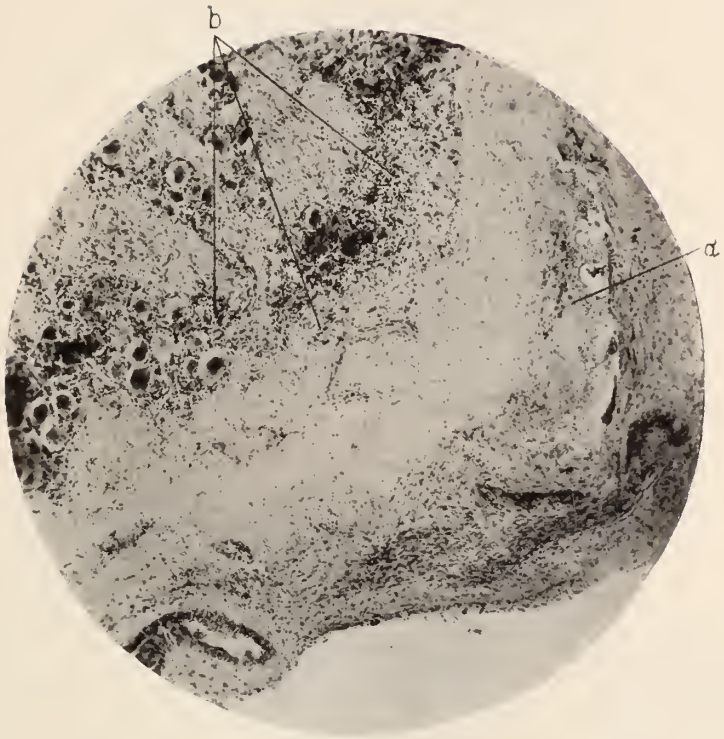
PLATE 16

Fig. 19

PLATE 17

Fig. 20
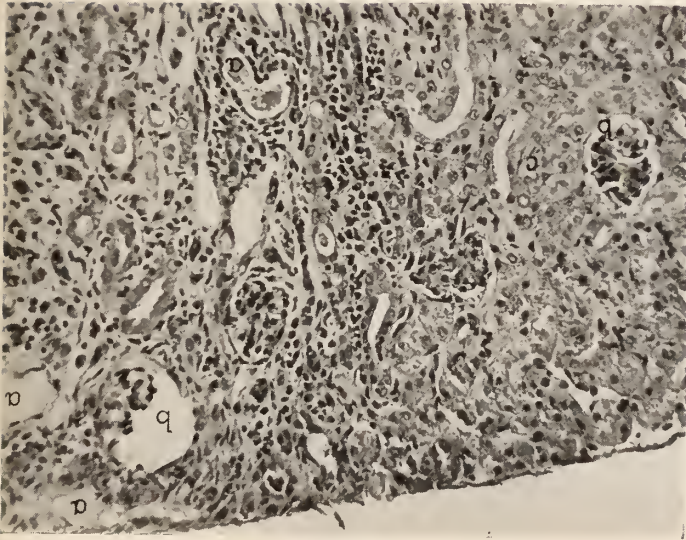
Fig. 21

Fig. 22

PLATE 18

Fig. 23.

F.g 24

PLATE 19
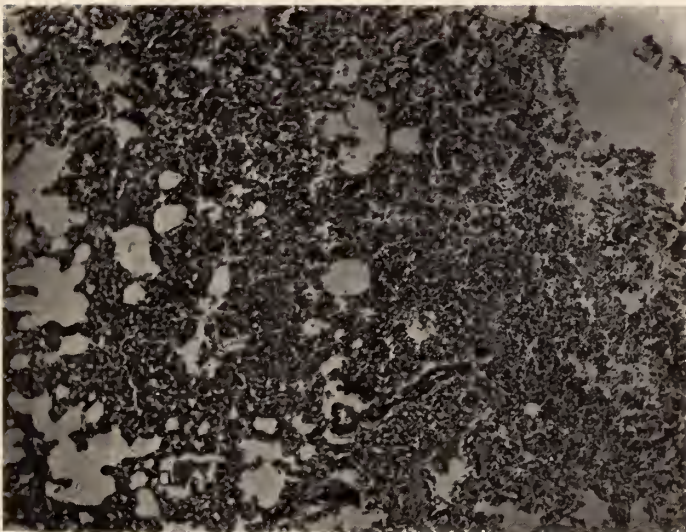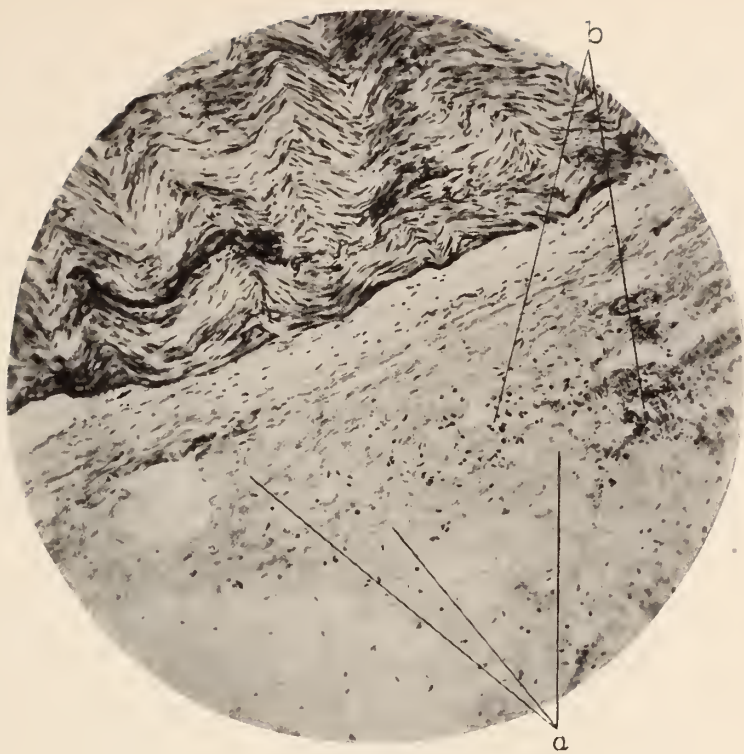
Fig. 25.

Fig. 26.

THE EPIDEMIOLOGY OF PELLAGRA IN NASHVILLE, TENNESSEE *

JAMES W. JOBLING AND WILLIAM PETERSEN

From the Department of Pathology of Vanderbilt University, Nashville, Tennessee

CONTENTS

INTRODUCTION

During the past two years the public health officers of the city of Nashville have had to deal with a startling and ominous increase in the morbidity and mortality from pellagra. In certain well-defined areas of the city particularly, the disease has been prevalent to an extent involving an economic loss of considerable magnitude quite apart from the physical suffering entailed.

The present facilities for the study and the relief of the situation available to any one of the public health bodies—state, county, or city—being inadequate, it was suggested that these officers co-operate with members of the department of medicine of Vanderbilt university. As a result, a committee met on July 25 at the offices of the city board of health and organized a pellagra commission

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constituted as follows: James W. Jobling, M.D., chairman; Olin West, M.D., of the state board of health, secretary; B. G. Tucker, M.D., county health officer; Wm. E. Hibbett, M.D., city health officer; and William Petersen, M.D. We wish to acknowledge our particular indebtedness to Dr. B. G. Tucker, whose extensive experience has been invaluable in the work of the survey, and in the preparation of this report.

Since no funds were available for this work, the voluntary assistance of several physicians connected with Vanderbilt university was secured. The members of the commission wish to acknowledge the generous co-operation of the following in this connection: Doctors Aycock, Briggs, Costen, Eggstein, Gregory, and Herbert, through whose efforts alone the present survey has been made possible.

Because of the limited number of workers and the lateness of the season (active work was commenced August 1), it was deemed expedient to make a survey of approximately only half of the city, including sections in all parts of the city—inclusive of the so-called residential parts as well as those districts inhabited by people economically less fortunate.

The members of the commission had no preconceived theory concerning the etiology of pellagra or of its mode of transmission. A questionnaire, as follows, was therefore prepared embodying all points which might throw some light on these phases of the subject.

QUESTIONNAIRE USED IN THE STUDY OF THE EPIDEMIOLOGY OF PELLAGRA

1. Name...Age......M. F., C. W., M. S., W. D.
2. Address..................... Street........................... Ward...........
3. Date of examination..
4. Examination of Patient.
    (a) Determine exact time of onset (month and year), the first symptom noted (whether gastro-intestinal, mental or skin change), and the sequence of these.
    (b) History and exact time of later attacks.
    (c) The present condition of patient and the type of symptoms.
    (d) Present occupation. Occupation during the past five years and at time of first attack. The present economic condition — earning capacity, number of dependents, etc.
    (e) Residence when first attack was noted (in detail); give details for the past five years, including street numbers.
    (f) History of previous illness (if severe). Evidence of hookworm?

5. Exposure to Disease.
    (a) Cases in immediate family. Onset of the first case? Type? Time of appearance and age of patient?
    (b) Cases among distant relatives? Contact with them?
    (c) Cases in immediate neighborhood or among friends?
    (d) Exposure due to occupation; visits?
    (e) Have there been previous cases in the same house?
    (f) What was the first case the patient has knowledge of?
    (g) How many brothers or sisters living? Dead?

6. Type of food.
    (a) Amount, number of meals, appetite?
    (b) Type of food? Protein, carbohydrate, fat?
    (c) Preference?
    (d) Is food properly prepared?
    (e) Change in type of food during the past five years?
    (f) Milk supply, name of dealer or source?

7. Type of Domicile.
    (a) House, number of rooms occupied, number of people living in house?
    (b) Type of sleeping quarters, number in room?
    (c) Frame, stone or brick?
    (d) Bath in house? How often used?
    (e) Toilet in house?
    (f) Screening?
    (g) Domestic animals?
    (h) Rats? Mice? Fleas?

8. Sanitation.
    (a) Connection with city sewage when illness began?
    (b) Open or closed privy? Distance from house?
    (c) Was privy used by other pellagrins?
    (d) Water supply. City? Well? Describe whether dug or drilled, bucket or pump,
        covered with wood or concrete. Spring? If from well or spring, give the exact
        address and location.
    (e) How garbage disposed?
    (f) Is there standing water on the premises?

9. Clothing and Light Exposure.
    (a) Is patient exposed to much light in occupation?
    (b) Kind of clothing. Colors of preference?
    (c) Shoes?

10. Personal Observations of Examiner.
    (a) Cleanliness of patient and home?
    (b) Insects? Body or head lice?
    (c) Apparent physical condition of family?

In addition, as each examiner went from house to house, he made note of the number of inhabitants, their race, and their approximate ages, and of the sanitary conditions, so that for the areas surveyed rather complete data are available concerning the population and sanitation.

In addition to this source of information there were available for study the records of the city, county, and state health offices.

Pellagra has been a reportable disease since 1911; the morbidity records are, however, of little use for statistical purposes for the following reasons: A considerable number of the cases are not seen by physicians; some are seen by physicians who even today insist that no such clinical entity as pellagra exists, and who persist in classifying and in treating these cases as "weeping eczema," "tetter," "seven-year itch," "nervousness," "amebic dysentery," "tabes mesenterica," etc.; certain other cases which occur in the more fortunate strata of the social organizations are purposely not reported because even physicians have gradually assumed an attitude of condonation toward the American habit of applying the law differently to the wealthy and to the poor.

The mortality records are undoubtedly of greater value, but err in that a large number of cases dying from pellagra have been recorded as dying from the complications or simple manifestations of the disease, no mention being made of pellagra itself. Even at the present time some 40 to 50% of the cases of pellagra are not so diagnosed on the death certificate. According to the statistics of the state board of health, about 600 cases of pellagra died during the year 1914 in the state of Tennessee.

In addition to the work outlined, the members of the commission have investigated several of the public institutions connected with the community life, and have made two additional surveys—one of a rural district in the same county of which Nashville is the county seat, and one in a small city about 40 miles from Nashville. Several etiologic studies have been conducted at the laboratories of the department of pathology of Vanderbilt university, of which a report will be issued later.

The members of the commission desire to express their appreciation of numerous courtesies from the members of the profession in Nashville and in particular from the following: Doctors Core and Lentz of the Davidson county asylum; Doctors McCabe, Manier, and Weaver of the city, Vanderbilt, and industrial school hospitals respectively; Doctors Douglas, Ragsdale, and Francisco of the Central state asylum; to Mr. Denton, chairman of the state board of control, and to Mrs. Boydston of the West Nashville free clinic.

It is the purpose of the commission to extend and complete the survey during the next spring and summer, the present report being published in the hope that the collection of data and the deductions that can possibly be drawn from them will be of sufficient value in view of the urgency of the situation and the growing interest of investigators and the medical profession as a whole in this disease.

DESCRIPTION OF NASHVILLE

Nashville, the capital of the state of Tennessee, is a city of approximately 115,000 inhabitants, about one-third of whom are colored. The city is located a little north of the central part of the state, lies on both banks of the Cumberland river, the older and chief commercial center being west of the river. This portion of the city is roughly divided into 3 parts; the northern part extending out into the lowland in the great bend of the river, the western part reaching to the river at the lowest segment of this bend, and the southern part of the city extending along the banks of the river and to the heights directly south and southwest of the city—Fort Negley, reservoir, etc. (see Chart 1). The city proper covers approximately 18.3 square miles.

Topography.—The city is built on a horizontal bed of limestone, the frequent outcropping of which, through the shallow subsoil, has given rise to the term "Rock City." To the south and west rise a series of hills forming part of the boundary of the central basin of Tennessee; these reach an altitude of possibly 500 feet above the level of the city and extend between the bends of the river previously referred to. From them small streams arise, which empty into the Cumberland; the valleys of these streams divide the city into 3 ridges, each terminating in a rock bluff on the river (Chart 10, p. 535). These streams—Lick, Wilson, and Brown's creek—form the basis of the sewage system of the part of Nashville west of the river. A single stream divides the eastern part of the city and forms the basis of its sewage system.

Distribution of Population.—The great bulk of the population lives in single detached houses and cottages of 3 or 4 rooms. The congestion of population in a small area in tenements such as occurs in the larger industrial centers of this country, does not exist in Nashville. The population is almost wholly native born, the greater part of the industrial class being drawn from the hill country immediately adjacent to the city. This element having been raised under conditions where sanitation is a negligible quantity (approximately

West

North

South

East

1910 ●
1911 ◉
1912 ◐
1913 ◻
1914 ▫

Chart 1. Ward map of the city of Nashville, showing morbidity from pellagra each year from 1910 to 1915, as reported to the city health office.

80% of the rural houses are even without privies) is quite content with the most primitive measures of sanitation provided in certain sections of the city. The white population is drawn almost wholly from older English, Scotch, as well as Colonial, immigration into the region from the Virginias and the Carolinas.

The colored population, numbering from 30,000 to 35,000, lives scattered over the city in rather well-defined groups, altho there is no legal segregation. The bulk of this population lives in the unsewered parts of the city, but their section of the city differs from that occupied by the whites in being at rather higher levels, which, despite the lack of sewerage, are well cleaned and flushed by every rainfall. The only exception is that part of the city known as "Black Bottom" (12th ward—Chart 2, p. 514), but in this low-lying area sewerage has been largely provided.

Economic Conditions.—The demand for labor is precarious, the wages are low and the family income is augmented, and in many instances almost wholly provided, by the labor of the children of the family. The opportunity for such employment is offered by several cotton and weaving mills, snuff factories, and similar establishments. More remunerative employment is offered for skilled labor in lumbering and woodworking, as well as in fertilizer mills, foundries, and railroad shops.

Economically, the average colored family is on a par with the poorer white families, for while the income may be somewhat less, the living expenses are lower in proportion, and the negro woman, while wasteful, is nevertheless frequently a good cook, so that the family is well nourished. The personal hygiene of the negro is equal to, and in some instances far superior to that of the average poorer white family.

Temperature.—The mean annual temperature of Nashville is 59 Fahrenheit, with an annual precipitation of 47 inches. The relation of morbidity to temperature is illustrated in Chart 9, on p. 533. It is true that the high temperature of the summer is of rather long duration and is occasionally very high (maximum 104); nevertheless the humidity during the summer months is usually low, so that heat prostration and death from such cause are practically unknown.

Water Supply and Sewerage.—The fundamentals of sanitation—water supply and sewage-disposal—have been provided in Nashville, but unfortunately for the health of the community, provided for only

part of the population, and of the two, the water supply has been extended to a greater number than the proper means of sewage-disposal.

The water is secured from the Cumberland river at a point about 4 miles above the center of the city, just below the entrance of Mill creek. After treatment with aluminum sulfate, sedimentation is permitted, and later further treatment with hypochlorite of lime is carried out. The reservoir is located in the southwestern portion of the city. This supply is augmented in certain parts of the city by numerous more or less superficial wells, which, unfortunately, are situated in those regions where soil-pollution occurs to the greatest extent. A number of springs are also highly esteemed in several of these regions; one of these is situated within 10 feet of an open sewer (Brown's creek) draining an area populated by some 10,000 people.

The sewerage system has developed from and follows the topography of the city, being entirely a gravity system. Because of the very shallow soil and the superficial nature of the limestone strata a rather unfavorable condition is brought about both in connection with the lack of proper filtration of surface waters and in the very considerable expense of the construction of needed lateral sewerage. The sewered area (Charts 10 and 11, pp. 535, 537) includes the older portion of the city, and, in a general way, the southern and southwestern parts of the city west of the river, and the areas of greatest density of population east of the river.

Even in the parts of the city where trunk sewerage has been provided, there are numerous blocks where lateral sewers are lacking and privies are used. It is reliably estimated that there are 12,651 privies in use within the corporate limits, practically all of which are in an unsanitary condition—only a very small number being screened. The fact that in one district alone (North) in an area of about 1 square mile 1500 privies were found (police survey), gives an approximate idea of the amount of surface-pollution. This area is of course that in which both typhoid and diarrheal disease and pellagra are found most prevalent (Charts 11, p. 537, and 13, p. 541).

HISTORY OF PELLAGRA IN NASHVILLE

Pellagra has given the public health workers of Tennessee grave concern since 1911, the year when the disease began to be reported from widely different sections with rapidly increasing frequency. It

was in 1908 that the first cases which had ever been definitely diag-
nosed as pellagra were seen in Davidson county, these having
occurred in the Baptist orphanage, an institution located just outside
the limits of the city of Nashville. In 1909 a case was discovered
in a man living in a house located on Hydes Ferry turnpike, a few
miles northwest of Nashville. This man died at the Davidson county
isolation hospital in 1909. An incident worth relating in connection
with this case is that a woman who lived next to him and who washed
his clothing developed pellagra in the year 1909. About the time
that this case was found, another man, residing in a community near
the corporate limits of Nashville called Flat Rock, was found with
a well-marked case of pellagra. All these cases were seen by Dr.
Tucker and Dr. West of the pellagra commission and by numerous
physicians in Nashville, to whose daily practice pellagra was a dis-
ease hitherto unknown.

The first case of which we can find record as having occurred in
the city of Nashville was a woman residing on Joseph street, in East
Nashville, in 1911. In this same year the first case of record was
found in North Nashville, but it was not until 1914 that the first
case was reported from West Nashville, this being the section in
which the Baptist orphanage referred to, was located and which has
now become a part of the city proper. In 1911 and 1912 reports of
pellagra in Nashville and in Davidson county began to be received
by the respective health departments in numbers sufficient to make
it apparent that the disease was rapidly becoming a very serious men-
ace to the public health.

Late in 1910 and early in 1911 reports were received at the office of the
state board of health in Nashville with such frequency as to indicate that pellagra
was becoming prevalent in a number of the counties of the state, principally
east and middle Tennessee counties. In the spring of 1911 the state board of
health added pellagra to the list of reportable diseases, and in July, 1911, adopted
a resolution advising the isolation of pellagra patients. At this time, also, a
pellagra commission, composed of three physicians, Wm. Krauss, B. S. Rhea, and
J. C. Brooks, was appointed to visit those counties in which it appeared that
pellagra was at all prevalent, to make such investigations as they saw fit, and
to offer such advice as they could give for the proper treatment and control of
the disease. This commission, during the summer of 1911, visited a number of
counties—about one-third of all in the state—and secured records of about 350
cases. It appears from their report, submitted later in the year 1911, that the
disease was most frequently found in the counties in east Tennessee and that
there was more of it in middle Tennessee than in west Tennessee. This seems
still to be true in 1916.

The cases at the Baptist orphanage in West Nashville were 17 in number and were all in children. It seems that 4 children of one family were received at the orphanage from a county of middle Tennessee in 1907. One of these children, a boy, probably had the eruption of pellagra when admitted to the orphanage. In 1908 the other children of this family group developed skin eruptions and were seen by Dr. J. M. King, of Nashville, to whom credit must be given for making a diagnosis of pellagra, a disease until then unknown in the experience of Nashville physicians. The diagnosis in these cases was confirmed in 1909 by Dr. C. H. Lavinder, of the U. S. Public Health Service, who was at that time in charge of the government work on the causation of pellagra. After the development of the cases in all the children of this one group, 13 other inmates of the orphanage were the subjects of pellagra, presenting well-marked skin symptoms, more distinct in some than in others. These children were isolated in a cottage and thereafter no other cases developed in the orphanage. Those affected were later removed to the Davidson county isolation hospital and 2 of them died. All the others recovered and, as far as is known to us, have remained well. One of these children later went to live in a home located some miles northwest of Nashville on the Hydes Ferry road and after the beginning of this residence 4 or 5 cases of pellagra developed in this immediate community.

While Dr. Lavinder was in Nashville, we had the pleasure of going with him on a visit of inspection to the Central hospital for the insane, the inmates of which are received from the counties of middle Tennessee. A thorough inspection of the patients was made and in none of them was there found any evidence of pellagra. A like visit to the Davidson county asylum, an institution in which indigent and insane persons are cared for to the number of 400 or more, failed to reveal any evidence of pellagra. No cases of pellagra in institutions in and around Nashville were reported until some time after the occurrence of the cases referred to.

Since 1910 one of us (West) has been in charge of the work in rural sanitation of the Tennessee state board of health, having from 3 to 5 associates engaged in field work in the various counties of the state. When this work was begun in the spring of 1910, the field men were instructed to be on the lookout for pellagra and to secure fecal specimens from every case, if possible, for the purpose of determining the coincidence of intestinal parasitic disease with pellagra

Dr. T. B. Yancey Jr., working in Knox county, found 5 cases of pellagra in that county in 1910, from which fecal specimens were secured for microscopic examination. Three of these were positive for hookworm ova, and as in other counties cases of pellagra were found in which hookworm infestation was found in about the same proportion (most of whom improved very markedly after successful treatment for hookworm disease and for disease due to other intestinal parasites), we were greatly interested in the subject of pellagra and made an effort to find as many cases of the disease as possible. Dr. Yancey heard of other cases in Knox county, but the disease was not so prevalent in the year 1910 as to cause the feeling of alarm which developed in the next year.

In 1911, in Knox county, Dr. Yancey, Dr. G. W. Booker, then health officer of Knox county, and one of us (West), went with Dr. Harrell to see a number of his patients who showed well-marked symptoms of pellagra. Dr. Harrell asked that we see his patients because his statement to the effect that he had 16 cases was disbelieved by other physcians in Knox county. There was no doubt as to the correctness of Dr. Harrell's diagnosis. While he had an unusual number of pellagra patients under his care, this incident well illustrates the rapidity with which the disease made its appearance in numerous homes after one or two cases had occurred in a community in a preceding year. Since 1910 pellagra has become more and more prevalent in Knox county each year.

One of the first cases seen by either of us in middle Tennessee outside of Davidson county was in a young woman, one of a family of 11, in Van Buren county in 1910. This woman was a patient of Dr. R. E. Lee Smith's, of Doyle, and hers was the first case which had occurred in his practice. Dr. Smith invited Dr. W. J. Breeding, then one of the field men of the state board of health, to see the case, and one of us (West) went to Van Buren county to investigate the diagnosis. This patient was very ill and none of us thought she would live more than a few days. The whole family of which she was a member were victims of heavy hookworm infestation. The woman with pellagra was given treatment for hookworm disease; she expelled several hundred parasites, and recovered her health. At our last report, in 1914, she was still well. This family was in very poor circumstances and it was not possible to have provided for the young woman a well-balanced and nourishing diet.

In 1911, when reports received by the state board of health began to indicate a rapid spread of pellagra, a report was received from Scott county of a family of 10 persons, all of whom were thought to have pellagra. Dr. J..A. Albright, then secretary of the state board of health, went to Scott county, saw these persons, and confirmed the diagnosis. Examination of specimens of feces from these cases showed hookworm ova in 8, and ova of Ascaris lumbricoides in 2 young children. Treatment for the expulsion of the intestinal para-sites resulted in bringing about a disappearance of all symptoms of pellagra.

In 1912 we saw the first case of pellagra which had been diagnosed among the inmates of the Tennessee industrial school, a state insti-tution near Nashville with about 800 inmates, received from all Ten-nessee counties. The patient was a boy. This case resulted fatally in 1913. In 1913 we saw the first case in the girls' department of this institution, in a girl from Fentress county. She was in a typhoid state and her recovery was not hoped for. She was removed to the Davidson county isolation hospital and, microscopic examination of the feces having discovered a heavy hookworm infection, was given thymol, with the result that her pellagrous symptoms quickly cleared up. A liberal nourishing diet was provided for this patient, but in spite of it the symptoms of pellagra reappeared in aggravated form after a short time. Another treatment with thymol effected the com-plete disappearance of all symptoms and after a few weeks the girl was returned to the school, where she now is and where her record has been a most excellent one. In 1913 several additional cases of pellagra occurred at the industrial school and it was feared that the disease would become prevalent. Isolation of all cases, however, seemed to stop its spread, for no additional cases developed that year. This experience recurred in 1914, isolation again apparently stopping the spread of pellagra after several well-marked cases had developed in that year. In 1915 fewer cases were noted in the school.

The Central hospital for the insane, the institution which was visited by Dr. Lavinder in 1909, has persistently refused to receive pellagrous patients. There were no cases in this institution, so far as is known, until 1914, except one, this patient having been sent home within a very short time after being received, when symptoms developed. In 1915 there were several cases of pellagra in this insti-

tution. Other hospitals for the insane in Tennessee have not been so strict in refusal of pellagrous patients and we are informed that each of them has had a number of cases. Dr. Core, superintendent of the Davidson county insane asylum, states that there were 2 patients in this institution in 1905 who presented typical symptoms of pellagra, but the disease was not recognized at that time. Since 1909, however, this institution has had a number of cases.

The history of pellagra in and around Nashville, and, for that matter, in Tennessee, has been made since 1907 or 1908. If pellagra occurred prior to those years, none knew its nature and certainly it was an extremely rare condition. At the present time, while the number of cases in Tennessee can only be guessed at, it is probable and practically certain that it is in the thousands.

As we have been engaged in public health work for a number of years, we have had abundant opportunity to see pellagra whenever it has been present in any considerable number of cases. In the years between 1900 and 1909—the latter year yielding, at the Baptist orphanage, the first cases of pellagra ever so diagnosed in Davidson county—neither of us had seen the disease. Since 1909, we have seen the rapid yearly increase which has brought the total number of cases in Davidson county to a figure that is most alarming. By reason of our duties in connection with the departments of health of Nashville and Davidson county, we were called on to vaccinate many thousands of persons during the years 1900 to 1910. In addition, we were called on daily to see patients in the practice of physicians of Nashville and Davidson county who had some form of skin eruption. We should be unwilling to make a definite assertion as to the number of persons we have vaccinated, but there could not have been less than 25,000 and we are confident that a much larger figure would do no violence to the facts. Smallpox was prevalent in Nashville in the very city wards in which pellagra has since become prevalent, and in several years the work of vaccination was carried on through the summer months; consequently, the fact that pellagra is a disease with a summer "up-curve" does not destroy whatever of value is to be attributed to any observations which we may have made. In all the thousands of instances in which we were called to vaccinate on the patients' arms or legs—the very parts on which the eruption of pellagra is most often found—neither of us can recall having seen any eruption resembling that with which we have since

become familiar as the eruption of pellagra; in all the many persons whom we were called to see with other physicians for the purpose of excluding or making a diagnosis of smallpox or of some other eruptive disease, we saw no case of pellagra prior to those seen in the Baptist orphanage in 1909; in visits of inspection to many industrial plants and institutions of various kinds, we saw no pellagra until 1909. Since then, and more especially since 1911, we have seen an annually increasing number of cases among the very classes, for the most part, among whom we had such abundant opportunity for finding the disease, had it existed, from 1900 until 1909.

It has been our observation that one case of pellagra in a community has practically always been followed by others, either in the same year or during the next season. The correctness of this observation is most certainly confirmed by the nature of reports from practically every community in which pellagra has appeared.

The morbidity from pellagra in Nashville as reported to the city health office for the years 1910 to 1915 is shown in Chart 1 on p. 505.

DISTRIBUTION OF PELLAGRA

The surveyed area included 14 of the 25 wards of the city together with a small area lying southwest of the city limits and forming part of the 8th sanitary district (Chart 2). This area is inhabited by approximately 65,000 people. Of these, 27,709 are adult whites and 14,114 are white children under 16 years of age. The adult colored population numbers 16,356, and the children, 6941. This population lives in 14,400 houses, making an average of 4.5 persons to each house.

The morbidity from pellagra found on examination was approximately 1 to 106 among the whites and 1 to 185 among the colored population; we have no doubt, however, that this approximation of the incidence is lower than the actual. It was impossible to examine all of the inhabitants, particularly males, who were usually at work when the examination was made; a certain number had had pellagra but in the absence of persisting skin changes or because of the unwillingness on the part of patients to give a history no diagnosis could be made; all doubtful cases were excluded; a certain number who had the disease either found it convenient to be away from their homes when it was expected that the examiner would call or refused

examination. This resulted to a certain extent from the fact that
the only place available for treatment has so far been at the county
isolation hospital, which, because of its inconvenient location and
because of the peculiar public attitude toward anything associated
with the term "contagious," led to a certain dread of the diagnosis
of pellagra, and a consequent evasion wherever possible. This atti-
tude of temporizing extended in a certain degree to some physicians
who were quite competent to make a proper diagnosis, but who in
frequent instances refrained from doing so, explaining away the
symptomatology on a basis which in some instances impeded the
recovery of the patient.

Chart 2. Ward map of the city of Nashville showing the extent of the pellagra survey.

From the survey it has become evident that the origin of pellagra
is not uniform throughout the population but is confined to certain
areas.

In the outermost tip of the 25th ward in a rather isolated com-
munity probably the highest rate is reached, some 10% of the popu-
lation here having developed pellagra (Charts 7, p. 529, and 11, p. 537).

North Nashville, comprising the 1st and 2nd wards (white) and
the 3rd and 8th wards (largely colored) was found to form a center
with a high morbidity.

In South Nashville (Wards 12, 13, 14, 15, and 16) pellagra is less evident, except at the outlying parts of these wards, where sewerage is either absent or deficient. Of the patients who have developed the disease in the sewered area very few have failed to give a definite history of frequent contact with cases elsewhere, or of having developed the disease in houses containing other pellagrins.
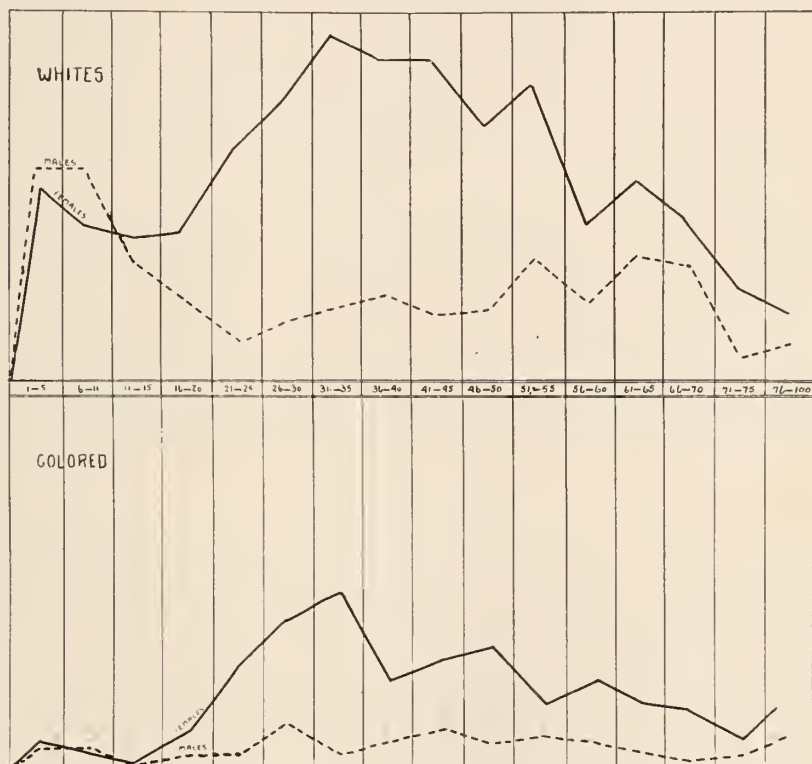
Chart 3. The incidence of pellagra according to sex, age, and color.

In East Nashville (Wards 17, 19, and 20) cases of pellagra were found grouped at the border of the sewered area, few of the cases having originated within that area.

INCIDENCE OF PELLAGRA WITH RESPECT TO AGE, SEX, AND RACE

Those who have investigated the subject, and especially the Thompson-McFadden commission,[1] agree that women are more sus-

[1] First Progress Report of the Thompson-McFadden Pellagra Commission, 1914, p. 29.

ceptible than men, and that children are less so than adults. This is
well shown in Chart 3, which gives the ages of 1021 cases. An
analysis of this chart shows that of 785 white patients, 249, or 31.7%,
were males, and 536, or 68.2%, were females. Of 236 colored people,
52, or 22%, were males, and 184, or 77.9%, were females. Both
sexes are apparently equally susceptible up to 15 years of age.

The Thompson-McFadden commission has called attention to the
apparent increase in resistance at the time of puberty, which is also
brought out in the chart. The greater susceptibility of women is
very evident both for white and for colored people after reaching
the age of 15, and continues throughout life, tho it is less evident
after the age of 55.

An interesting series of cases was that occurring among nursing
infants. Twelve pellagrous babies have been observed during the
past few years, and of these 5 had pellagrous mothers.

Chart 4, giving the mortality of the series of 1021 cases mentioned,
shows that the death rate corresponds very closely with the curve
of age incidence shown in Chart 3. The resistance to infection dis-
played at the time of puberty is shown here by the fact that there
was only one death at this age, and that was a colored boy.

TIME OF ONSET

It is well known that pellagra develops more frequently during the
warmer seasons of the year and this is shown graphically in Chart 5.
An interesting fact brought out in this chart is that negroes do not
appear as susceptible as whites during the months when the rays
of the sun are not so direct. The figures show that 50% of the
white patients developed the disease during the spring months as
compared with 30% of the negro patients, while 44% of the white
patients developed the disease during the summer months as com-
pared with 66% of the negroes. Of the white patients, 5.8% had
their first attack during the autumn and winter months, while 3.2%
of the negroes first developed the disease during the autumn, and
none during the winter months.

These data are of interest as the theory has been advanced that
pellagra is due to photodynamic substances.[2] If such were the case,
we should expect that negroes, because of their dark skin, would not

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[2] Raubitschek: Wien. klin. Wchnschr., 1910, 23, p. 23.

be as susceptible to the rays of light as white people, and that a longer exposure would be required.

By the use of the term "first attack" we mean when the patient first became actually ill, presenting those symptoms which are considered

Chart 4.   The mortality from pellagra according to sex, age, and color.

as pathognomonic of pellagra. It is probable, however, that the patients had had the disease for some time previous to the acute attack, as the majority stated that they had been suffering for weeks,

and sometimes for months, with nervousness and with symptoms referable to the gastro-intestinal tract.

DURATION OF THE DISEASE

At times neither the patients nor their friends were able to state when the first symptoms of the disease had appeared, and so in Chart 6 the histories of only 493 cases are shown.
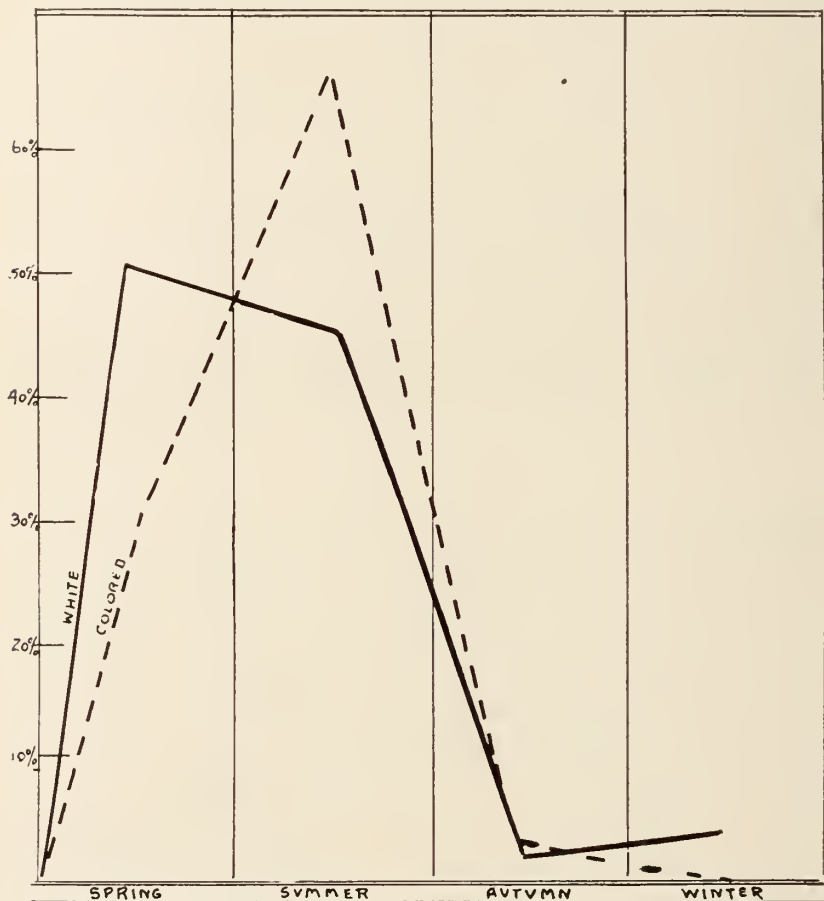
Chart 5.   The seasonal onset of pellagra among white people and among colored people.

It will be seen that 234, or 47.6%, of the cases developed during the spring and summer of 1915, and 70, or 14.1%, in 1914. From then back to 1906 the cases became progressively fewer. We have
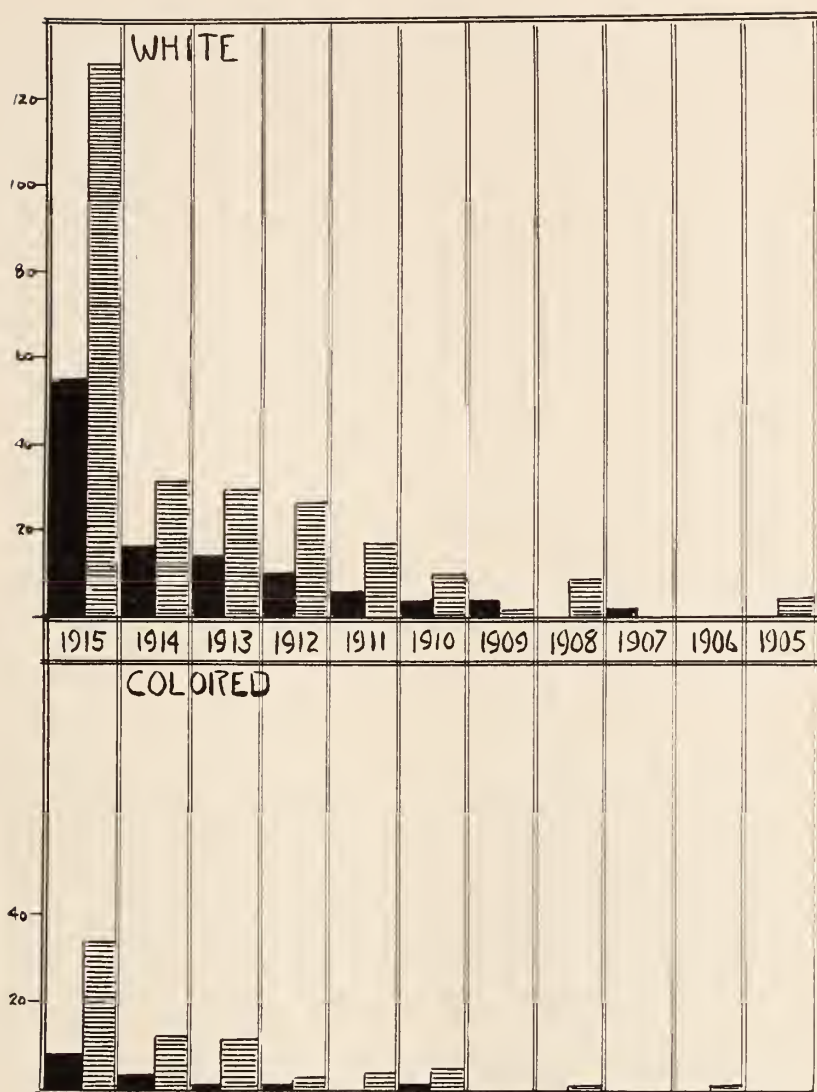
Chart 6.   Duration of pellagra.

records of 12 patients, 11 of whom are still alive, who developed the disease previous to 1906, and of one who had her first attack in 1891.

From the data given, one of two conclusions may be drawn: first, that the number of people attacked is becoming much greater, and

second, that the death rate for the past few years has been much higher than our figures indicate. It is probable that both factors are concerned and that the disease is not only more prevalent, but that the death rate has been much higher than is shown in our reports. It must be remembered, however, that these data deal only with those of whom we could get a definite history—493 cases—and not with the entire series. An analysis of the other cases might show a greater prevalence in the years preceding 1915.

The majority of the patients who had had the disease for 5 or more years had not had an attack each succeeding year. Frequently there had not been more than 2 or 3 attacks during the interval, and in one instance, the patient had had a severe attack in 1908 and none after that.

The inquiries devoted to determining the time of onset of each case not only brought out the fact that the disease has possibly been present in this locality since 1891, but they also gave us much added information concerning the relation these cases bore to succeeding ones. In the majority of instances, as will be discussed later, pellagrins were shown to have been closely associated with individuals who subsequently developed the disease.

RELATION OF PELLAGRA TO DIET

Special data could not be obtained for all the 501 cases composing this study, but information which we believe to be reasonably accurate was obtained concerning 421 patients, of whom 320 were white people, and 101 negroes.

The belief that pellagra is due to an improper diet has received much credence, and Goldberger[3] and his associates in their experimental studies of several orphanages in the South have shown that a properly balanced diet is an important aid in preventing the annual recurrences of the disease. However, before this can be recognized as the sole etiologic factor causing pellagra, it must be shown that the diet of the people in the localities where the disease has become so prevalent during the last few years has been changed for the worse.

For these reasons inquiries were made of the patients and of their friends as to whether there had been any definite change in

[3] Pub. Health Reports, 1915, 30, p. 3117.

the general character of food consumed during the 2 years previous
to the onset of the disease, and whether there had been times during
this period when they had not had sufficient food. Of the 320 white
people, 46, or 14.4%, stated that there had been times during the 2
years preceding their first attack when they had not had sufficient
food, and of the 101 colored people, 12, or 11.8%, gave a similar
history. This statement of course applies to a deficiency of all con-
stituents and not to a deficiency of any particular one. In only 5
instances were we able to obtain information that there had been a
definite change in diet for the worse during the 2 years previous to
the onset of the disease. In the remaining instances the patients
and their friends asserted that the food consumed had been the same
or better in both quality and quantity than they had been accustomed
to previously.

Practically all the patients were consuming an excess of carbohy-
drates. This excessive use of carbohydrates has been discussed
already by Goldberger[3] and by the Thompson-McFadden pellagra
commission.[4] It appears to be rather general throughout the South.
Corn in the form of corn-bread and grits is used extensively, tho
corn-bread is not used so much among those who have lived in Nash-
ville for some years as among those living in the rural districts. It
is, however, used frequently by the poorer people; bread, in the form
of biscuits, made of wheat flour, is also used a great deal. Molasses
made from sugar cane enters largely into the diet of the poorer people,
tho from statements made it would seem to be consumed chiefly by
the children. The last statement does not apply to pellagrins, as a
number of patients were living on bread and molasses at the time
they were discovered. Investigation, however, revealed the fact that
this was not their usual diet, but that it had been adopted because
they had a "nervous dyspepsia," which, they believed, was aggravated
by meat. In the spring, summer, and autumn months a great deal
of green stuff in the form of turnip-tops, wild mustard, green peas,
and green onions is eaten. The turnip-tops and wild mustard are
cooked with fat meat—sow belly—but the green onions are eaten
raw. In addition, much fruit, especially apples and peaches, are eaten
during the summer months, as these fruits are usually cheap—from
15 to 20 cents a peck—during this period of the year.

[4] Siler, Garrison, MacNeal: Arch. Int. Med., 1914, 14, p. 293.

The view that pellagra is due to a deficiency of protein in the
diet has received considerable prominence in this country. However,
just what constitutes a protein deficiency sufficient to cause the dis-
ease has not been stated, and so we must resort to the data supplied
by Voit,[5] Chittenden,[6] Attwater,[7] and others, and from this infor-
mation determine whether the patients had been receiving a sufficient
amount of protein to sustain them.

We are not in a position to say just how much protein is neces-
sary for the maintenance of life. Voit[5] believes that 118 gm. of pro-
tein, of which 105 gm. must be digestible, are necessary for an indi-
vidual weighing from 70 to 75 kilos. Chittenden,[6] however, with a
number of his assistants, several athletes and soldiers, showed that
this amount of protein is not necessary; Chittenden kept himself in
a condition of nitrogenous equilibrium for months on from 37 to 40
gm. of protein a day. McCay,[8] in his studies of the metabolism of
the Bengalis, of Calcutta, India, found that the natives consumed an
average of 40 gm. of protein a day, of which from 2 to 5 gm. were
in the form of animal protein. If 40 gm. of protein a day is the
average for these natives, it is certain that many must receive still
less, and yet we hear no mention of the prevalence of pellagra.
McCay sought to determine the influence of low protein intake on
the health and economic conditions of the people. He states that they
are tall, but that the chest measurements are less than those of Euro-
peans, and that their muscular systems are underdeveloped. He offers
this as the explanation of their inability to accomplish as much manual
labor as Europeans. He believes that diseases of the kidney occur
more frequently among these natives than among Europeans, but
makes no mention of pellagra.

It seems that we are justified in assuming not only that a diet
containing 40 gm. of protein is sufficient to sustain life, but that, in
view of the experiences of Chittenden and his assistants, and the
work of McCay, such a diet does not produce pellagra. This then
gives us a basis on which to work; in other words, gives us what
may be called a limit of safety.

In discussing the amount of protein necessary to sustain life we
must not forget the work of Mendel and Osborne.[9] They have shown

[5] Physiologie des Stoffwechsels, 1881, p. 519.
[6] Physiological Economy in Nutrition, 1904.
[7] Principles of Nutrition and Nutritive Value of Food, 1906.
[8] Scientific Memoirs, New Series Medical and Sanitary Department, India, 1908, 34.
[9] Jour. Biol. Chem., 1914, 17, p. 325.

that the character of the protein is as important, if not more so, than the quantity, as some proteins are not able to sustain life in experimental animals even when given in excess.

Of the 421 patients included in this series, 282, or 66.8%, gave histories which indicate that they had been getting considerably more than 40 gm. of protein a day. According to races, 66.8% of the white people and 67.3% of the colored people consumed more than this amount of protein daily.

Some of the patients consumed even more than Voit recommends. One family, in particular, shows this fact very well. The family moved from the country to South Nashville and opened a grocery store and butcher shop. Two years later a married daughter who was suffering from pellagra came from the country to live with them; the following spring another daughter and her cousin, who lived in the same house, developed the disease. Careful inquiries concerning the diet of these two children brought out the fact that they had been getting eggs with ham and bread for breakfast, fresh meat for dinner, and all the milk they desired during the day, and besides, according to the father, they would slip into the store and steal bologna sausage, dried meat, candy, etc. In another instance, a colored girl, whose parents owned a grocery store and considerable real estate, developed the disease in spite of the fact that she had been eating fresh meat, milk, and eggs daily. These cases, however, are exceptions, as the diet of the poorer people of Nashville contains an excessive amount of carbohydrates and less protein than that of northern people.

It is possible that the deficiency in protein may be in quality and not in quantity. Mendel says that we should speak of "amino-acid minima instead of protein minimum." This possibility cannot be denied in certain cases, but the diet of the majority of the patients was sufficiently varied to make it improbable. The fresh meat used most commonly among the poorer people is shoulder meat from beef, probably because it is the cheapest. Beans form rather an important part of the diet; ham is eaten by some, and practically all consume a large amount of fat pork meat. In addition, some drink a great deal of butter-milk, which is purchased almost daily, as it is used in the preparation of biscuits and corn-bread. One woman with a well-developed attack of the disease stated that she could not remember a time for years when she had not had at least one glass of butter-milk at each meal.

According to Mendel and Osborne,[9] it appears that one of the first effects of insufficient protein on young animals is the inhibition of growth. Mendel[10] states that "adequate growth postulates a satisfactory condition of maintenance before any continued gains in

[10] Harvey Lectures, 1914, 15, p. 101.

weight can be made." If this holds true for human beings and if pellagra is due to a deficiency of proteins, or to other dietary faults, we should expect that children who suffer from this disease, and those who live in such families, would show evidence of stunting. On the contrary, no such influence on growth was noted either in the children of families containing pellagrins or in those with pellagra. In size they compared favorably with those in the districts of the city where pellagrins were not found, and not infrequently the disease developed in those who appeared most sturdy.

Another peculiar thing from the standpoint of protein as the causative agent is the fact that men, with the same diet as women, yet with a protein requirement probably greater than that for women, are less susceptible.

RELATION OF PELLAGRA TO SANITATION

Wherever cases of the disease were found, careful inquiries were made concerning the methods of disposal of the excreta, and the source and character of the water used. Trustworthy information was not always to be obtained on these points when the onset of the disease had occurred at some distant place, especially when the patient had been ill for more than a year; hence, we are compelled to rely on the data obtained from a study of 501 cases.

This group of 501 cases was composed of 394 white people and 107 colored. Of the total number, 442, or 88.8%, first had developed the disease while living in houses which were not connected with sewers, and some of the others had presented the first symptoms while living in localities where sewers had only recently been installed. Moreover, some few of the cases which had developed in houses connected with sewers had been either just across the street from, or in houses adjoining, other pellagrous dwellings which had no sewer connections. According to races, 88.2% of the white people and 85.9% of the colored, had developed the disease in houses not connected with sewers.

A classification of the privies used by pellagrins according to the method used by the Rockefeller sanitary commission would be useless here, for while some used the pail system, the benefit which might have accrued from its use was nullified because the pails were almost never protected from flies. In addition, the outside of the pails was almost as filthy as the inside, and from their battered appearance, it

is probable that very few were water-tight. The pails both inside and outside were usually covered with flies. In many instances the excreta of the open-surface privies were disposed of by chickens and hogs. Of the 442 cases, only 20, or 4.6%, were in houses where the privies were properly screened.

The privies are rarely more than 50 feet from the back of the house and frequently not more than from 15 to 20 feet from the kitchen. Not more than 2% of the houses were screened; therefore it will be seen that the flies, which were always very numerous around the privies, could readily pass back and forth from the kitchen to the privy. This is also the probable explanation of the large number of cases of typhoid in the same localities, but this phase of the subject will be discussed more in detail later.

The water used by these patients was obtained from the regular city supply, from wells, cisterns, and springs. The city water supply is obtained from the Cumberland river. The intake is located about 4 miles from the city at a point not far distant from the outlet of Mill Creek, a potential source of pollution at certain stages of river level. The water is treated with aluminum sulfate and sedimentation permitted. It is later treated with calcium hypochlorite. The low bacterial count shows that it compares favorably with that used in other cities.

The cistern water is not as good as might be expected because of the fact that many of the cisterns have cracks in the walls permitting the entrance of surface water, while the majority were not properly covered, and thus acted as breeding places for the mosquitoes, which are present in great numbers. The thin subsoil and the underlying bed of limestone with its cracks and crevices, render the water obtained from shallow wells and springs unfit for use, as no opportunity is presented for filtration. This is particularly true where the wells are only a short distance from the privies.

The same criticisms can be made of the springs, which are probably a greater menace because of the greater number of people using the water. All the water, except that from cisterns, contains considerable lime, and is hard water.

An analysis of 501 cases shows that 55% of the patients used city water, 1.2% spring water, 3% cistern water, and 40% well water. This indicates that the source of the water has but little to do with the development of the disease.

Nashville does offer some very positive evidence in regard to one theory concerning the etiology of pellagra—the theory of Allessandrini.[11] According to this hypothesis, the drinking of water containing silicates in certain concentration causes an acidosis which results in the symptom complex of pellagra. Water from limestone sources, on the other hand, is supposed to be remedial. A considerable number of pellagrins in Nashville have always used such water and have developed the disease while drinking it.

RELATION OF PELLAGRA TO PREVIOUS EXPOSURE

In the study of a disease like pellagra, the morbidity and mortality of which are increasing rapidly, we must determine not only the local conditions surrounding each patient, but also the relation which each case bears to preceding ones. In other words, as long as the etiology of the condition is obscure, each and every phase of the subject should be gone into fully, with unbiased mind.

Whenever a case of the disease was found, endeavors were made to find whether there had been other cases in the house or in the immediate neighborhood. Frequently when we were unable to find other cases in the neighborhood, subsequent information obtained from Dr. B. G. Tucker, the county health officer, whose extensive experience with the disease during the past 8 years has made him familiar with most of the cases, showed that the patients had intentionally or unintentionally deceived us, and that they had been in intimate contact with other pellagrins. Moreover, not a few of the patients had been in the habit of paying long visits to friends suffering from the disease. The residences for the preceding 5 years, especially after the development of the disease, were obtained from each patient, and when these had been marked on the map, it was found that a number of the patients who asserted that they had never heard of the disease before, had been living in houses adjoining others which contained pellagrous patients.

That a close relation exists between each case and those preceding it, is shown by the following figures: Of the total number of cases, 169, or 33.7%, developed in houses which contained other cases, while 94, or 18.7%, developed where there were cases in the adjoining houses. Of the remaining patients, 97, or 18.3%, lived in the

[11] Annali d'Igiene, 1914, 24, p. 1.

same block with other patients with whom they associated, while 35, or 6.9%, visited patients elsewhere, or were associated with them in their daily occupation. Combining these we find that 394, or 78.8%, of the pellagrins, both colored and white, had been associating with other pellagrins.

Of the two sexes, 267, or 78%, of the 342 females, gave histories of having previously associated with other patients, and 128, or 80.5%, of the 159 males, gave similar histories. From this it will be seen that the two sexes had been equally exposed, but it must be borne in mind that the males constituted only 31.7%, or one-third, of the 501 cases composing this series.

The significance of these figures will be better realized when one stops to consider the difficulty encountered in obtaining contact histories with such a disease as typhoid fever.

A marked difference was found between the number of contact histories obtained of white people and the number obtained of colored people, the figure for the white people being 84.5%, and that for the colored 57.9%. This is probably due to the difficulty encountered in getting good histories from the colored people because of their failure to remember dates and addresses. Both classes among the poorer people move frequently, but while the white people can usually give the exact addresses of their previous residences, the negroes can rarely do so.

RELATION OF PELLAGRA TO THE DENSITY OF POPULATION

The origin of pellagra in Nashville bears no relation to the density of population either of whites or of colored people.

If pellagra is transmissable and due to a constant contact, we might assume that the opportunity for such contact would be much greater in the area of greatest density of population. This factor is nullified to some extent, however, by the fact that in the districts where the population is not so dense, the house to house association due to constant visiting with neighbors—which becomes apparent in survey work of this kind—may be much more intimate than in the tenement districts where two- and three-storied houses enable more people to live in a small area.

The relation is further complicated in that the conditions as regards sanitation are not comparable, because the area of greatest density

is practically wholly sewered. Economically, at least as far as the
surveyed area is concerned, there is no great difference between the
population in the area of greater density and that in the area of less
density.

The approximate ratio is illustrated in Chart 7, in which relative
density of the separate wards is expressed in various degrees of shad-
ing. The relation of density of population to the morbidity from
pellagra is also shown in Chart 7.

RELATION OF THE DISEASE TO SOCIAL AND ECONOMIC CONDITIONS

A study of the epidemiology of pellagra in a community would be incom-
plete without an investigation of the economic conditions. For this reason, we
tried to obtain information concerning the wages received and the general cost
of living for the past 5 years in Nashville. On this subject, however, we were
unable to obtain accurate information; therefore, the statements which follow
must be more or less general in character.

The wages received today by the class of people among whom pellagra is
most prevalent are somewhat higher than those received 5 years ago. Work
was readily obtained until the early autumn months of 1914, when as a result
of the financial crisis incident to the European war, some factories were closed,
others were run only part of the time, and the average wage of the laborer
was therefore reduced. This condition resulted in a slight increase in the
number of people making application for assistance to the charitable institu-
tions during the winter of 1914-1915. In the spring of 1915 work became more
plentiful and by September the factories were all running full time.

House rents in the surveyed areas have not increased during the past 5 years.
This holds true for those having vault and surface privies, as well as for those
having the alley sewer connections.

Food, especially meat, has increased in price during the past 10 years, but
the increase has been comparatively little during the past 5 years. In 1913
vegetables were also high owing to a failure of the crops, but in 1914 and
1915 they were plentiful and cheap. During the summer of 1915, when pellagra
was more prevalent than ever before, vegetables were very cheap.

It appears then that there was a period of economic depression beginning
about September, 1914, and lasting into the early summer of 1915. This depres-
sion was made evident by the increase in the number of applicants for assist-
ance at the charitable institutions. This depression was partly neutralized
by the cheapness of certain foods. The first great increase in the number of
cases of pellagra, however, occurred in the spring and summer of 1914, before
the outbreak of the war; therefore, the increase cannot be ascribed to this cause

In discussing the influence of periods of economic depression we should not
forget the years 1893, 1903, and 1907, when conditions in Nashville were at
their worst, and yet no pellagra was reported.

THE MORTALITY FROM PELLAGRA

During the years from 1910 to 1915 there occurred in Nash-
ville about 100 deaths in which a diagnosis of pellagra was recorded

Chart 7. The relative density in population of the various wards and the origin of 600 cases of pellagra therein.

on the death certificate or entered as the chief or as a contributory cause of death. These are distributed as follows:

1910.. 8
1911.. 12
1912.. 6
1913.. 16
1914.. 20
1915.. 37
                                            ──
                                            99

This, however, does not include the patients from Nashville who died at the isolation hospital. When these are added the figures are as follows:

1910.. 8
1911.. 16
1912.. 14
1913.. 23
1914.. 24
1915.. 62
                                            ──
                                            147

Apart from these recognized cases, numerous deaths occurred from pellagra in which the proper cause of death was not stated on the death certificate. These were ascertained as follows: The individual morbidity records were compared with the death records, and when it was found that a case had been reported by some physician as pellagrous with a later fatal termination, the symptomatic diagnosis usually recorded was disregarded and the death considered as due to pellagra; certain of the cases had been seen by the county health officer and by the city health officer, and, tho the death certificates did not properly record the cause of death, it was regarded as a death from pellagra; in a few instances, probably less than a dozen, a clear history was obtained from relatives. During the year 1915, of course, a considerable number of cases seen by our examiners terminated fatally. In such instances, if the death certificate did not record the death as due to pellagra, we made the proper notation on our records.

With these additional records the mortality rate was corrected and for the 6 years is approximately as follows:

1910.. 28
1911.. 54
1912.. 40
1913.. 47
1914.. 55
1915.. 108
                                            ──
                                            332
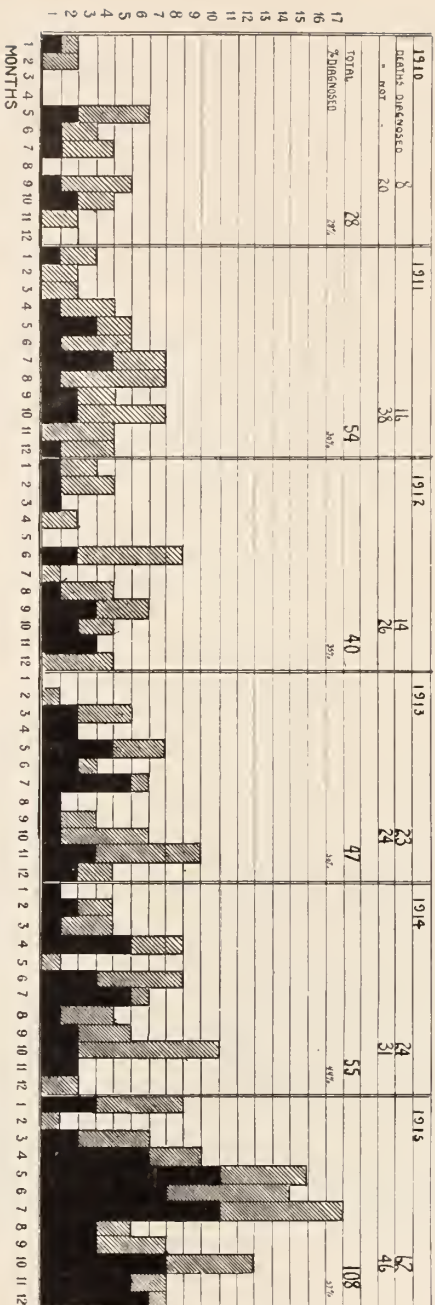
Chart 8. The black columns indicate the number of cases resulting fatally each month (1910-1915) that were diagnosed as pellagra; the shaded columns, those which were not so diagnosed.

About 46 other cases are known to have terminated fatally, some of them prior to 1910; the greater number of them originated in Nashville, but because it was impossible to trace the death records of some of these, they have not been entered in the tabulated list.

Of the total number, 262 were white and 116 colored. Of the 262 whites, 77 were males and 185 females. Of the 116 colored, 31 were males and 85 females. The ages at the time of death are shown in Chart 4 on p. 517.

Of the 332 cases that have resulted fatally since 1909 it will be recalled that a considerable number were diagnosed. The proportion is brought out in Chart 8, in which the black columns indicate the number of cases resulting in death each month which were diagnosed as due to pellagra, and the shaded columns the cases which were not so diagnosed. It will be observed that the percentage of cases diagnosed has increased progressively, so that at present only about 40% of the total number are not properly recorded on the death certificate.

Among the causes of death which were assigned on the various death certificates, it might be of interest and of some value to enumerate the following:

Gastro-intestinal	Dysentery, flux, diarrhea	41
	Tuberculosis of bowels	17
	Tabes mesenterica	
	Iliocolitis	15
	Gastritis-gastric catarrh	15
	Cancer of stomach	6
	Cirrhosis of the liver	3
Oral	Noma	
	Ptyalism	
	Cancrum oris	8
	Stomatitis	
Nervous	Neurasthenia, melancholia	
	Menopause, dementia	29
	Insanity, meningitis	
	Suicide	5
	Heart disease, grip	13
	Typhoid, senility	
	Nephritis, tuberculosis	

Occasionally several of the more or less symptomatic diagnoses were found entered, apparently because there was some question in the mind of the physician. At other times skin lesions were also added, such as "tetter," "eczema," "erysipelas," etc.

The death rate for 1915 approximately doubled that of the previous year. It is difficult to determine whether or not this increase had some relation to the unfavorable economic condition which prevailed during 1914. Such a relation is at any rate a factor that must be considered.

The relation of the total mortality to the mean monthly temperature for the 6 years is illustrated in Chart 9. The curve shows 2 periods
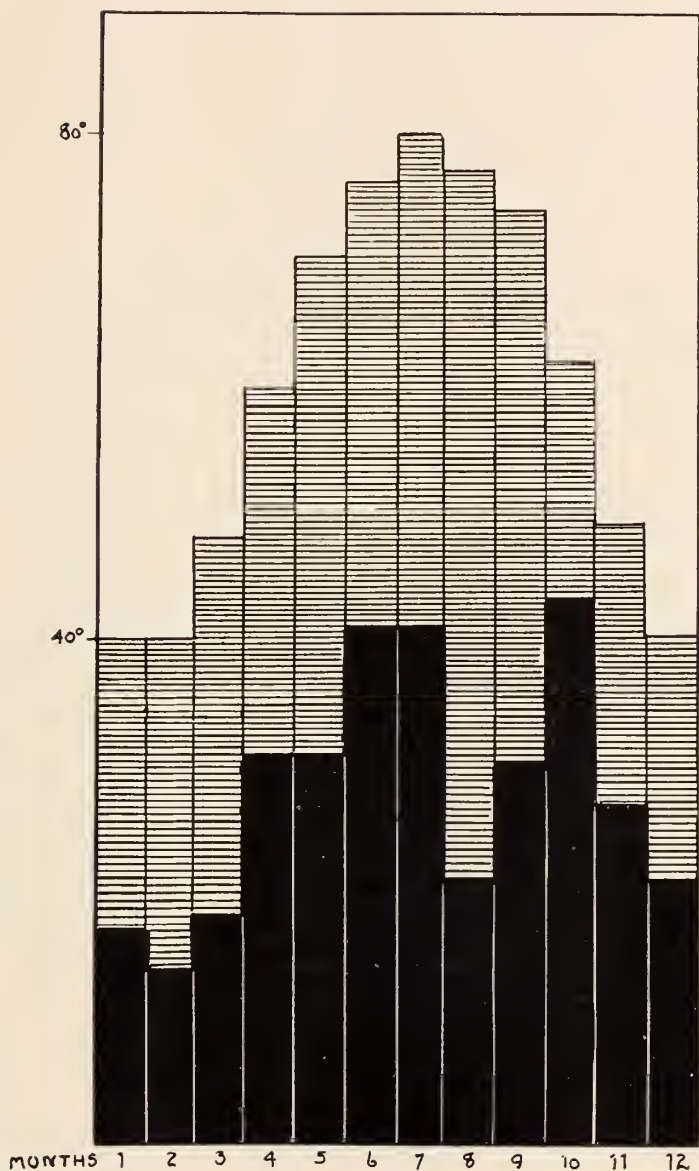
Chart 9. The relation of the mortality from pellagra to the mean monthly temperature for a period of 6 years.

of increased mortality, one during June and July, the other during
October. It seems that the first period of prolonged high temperature
kills off the most debilitated cases carried over from preceding years;
then, with the exhaustion of this material, the mortality is decreased
until such time as the more severe cases which have originated during
the same year have commenced to succumb, usually late in the summer.

THE RELATION OF PELLAGRA AND TYPHOID TO THE SYSTEM OF SEWAGE-DISPOSAL

In the preceding discussion it has been shown that fully 88%
of the cases of pellagra which have originated in Nashville have
originated in houses without facilities for sewage-disposal other than
open privies. A very evident relation might therefore be presumed
to exist in this connection.

In order to obtain a more comprehensive idea of the conditions
responsible for the present status of the sewage plant it is necessary
to refer to the topographical map (Chart 10).

In the part of the city lying west of the river 3 drainage areas
are established: (1) Lick Branch, draining through the so-called
Sulphur Dell and providing sewerage for the greater part of the city;
(2) Wilson Branch, draining through the lowland known as Black
Bottom; and (3) Brown's creek, an open sewer to which the entire
southwest drainage of the city is diverted. In East Nashville the chief
drainage area is that occupied by the so-called Edgefield sewer (4).
In addition, several accessory sewers have been constructed, entering
the river at various points, as will be noted in the diagram of the trunk
sewers. This trunk system provides drainage for the greater part of
the city, but excludes the westernmost and northern parts of the city,
and the hill areas, where the nature of the ground (limestone) makes
sewer construction both difficult and expensive. The existing trunk
sewers give, however, only a rough estimate of the actual area provided
for as regards water-conveyed sewage; for in some instances the
sewers are at a level higher than the toilets to be flushed, serving
purely as storm sewers, to carry off excess rain-fall; in some parts of
the city even this provision is inadequate. In the poorer section of
the city when sewerage is provided, the conditions are by no means
ideal, for the toilets using so-called alley sewers, are out of doors and
in the majority of instances in an exceedingly filthy condition.

Chart 10. Topographical map of Nashville, showing the existing system of trunk sewers.

The value of the trunk sewer is impaired because the essential lateral sewers have not been completely constructed. In a part of South Nashville (13th and 14th wards) this condition was only remedied during very recent years and the area still suffers from a rather high typhoid morbidity. On the other hand, in some instances sewerage has been provided for some blocks, while water connections are lacking. During the last year some blocks have been sewered in North Nashville, which until the present year furnished large numbers of pellagrins. The actual sewered area is shown in Charts 11, 12, and 13, in which the shaded area represents the part of the city in which sewerage connections are possible.

When we now consider the sewered area, it is found that as regards the relation of pellagra to the disposal of water-conveyed sewage 4 groups of wards can be studied (see Charts 2, p. 514, and 11, p. 537).

Group I (North Nashville), containing the 1st, 2nd, 3rd, and 8th wards, is largely unsewered.

Group II (West Nashville), containing the 24th and 25th wards, is wholly unsewered.

Group III (South Nashville), containing the 12th, 13th, 14th, 15th, and 16th wards, is, with the exception of the 15th ward, now fairly well provided with sewers.

Group IV, containing the 17th, 19th, and 20th wards, is also fairly well sewered, the areas indicated as not yet sewered, being largely factory sites.

We can therefore roughly divide the sewered areas into 2 sections, a poorly sewered area (Groups I and II) and a fairly well-sewered area (Groups III and IV).

In the 1st section, lives 46% of the total surveyed population; in the 2nd section, 54%.

In the 1st section, 10,100 negroes live, in the 2nd, 12,100, the approximately equal population indicating approximately equal economic standards in the two divisions. It should be emphasized that, apart from the evidence afforded by the apparently equal distribution of the negroes, the two divisions are representative of equal economic and social conditions in other respects.

In these areas approximately 600 cases of pellagra are known to have originated (about 375 during the past 2 years) and about 435 cases of typhoid have originated in this area during the past 2 years.

The percentage of known cases of pellagra originating in these divisions, the percentage of cases originating during the past 2 years,

Chart 11. Relation of the morbidity from pellagra to the unsewered areas. The shaded portion indicates the part of the city in which sewerage connections are possible.

the percentage of deaths from pellagra, and the percentage of morbidity from typhoid are shown in Table 1.

An analysis of the table reveals the following facts: In the 1st division (inadequate sewerage) with 46% of the population, originated 63% of all cases of pellagra, and of the cases developing during the past 2 years, 69%. The corresponding figures from the 2nd division (fairly well sewered) are 35 and 31% respectively.

These figures, apart from their direct bearing, would also indicate that in the area with the poor sewerage the tendency is toward an increase in the number of cases. Whereas in the 2nd division, which has had considerable improvement in sewerage during the past 5 years, pellagra is decreasing at present. It is to be emphasized, however, that even in this 2nd division surveyed, in which the sewerage is fairly adequate, the majority of cases of pellagra originating occurred in houses which were without sewerage connections.

TABLE 1

Relation of Pellagra and Typhoid to the System of Sewage-Disposal

Sewer System		Percentage of Total Pellagra	Percentage of Pellagra 1914-15	Percentage of Deaths from Pellagra	Percentage of Typhoid Morbidity
Inadequate......	Group I (Wards 1, 2, 3, 8)	36	40	35	43
	Population 21,909 (34%)				
None............	Group II (Wards 24, 25)	27	29	16	13
	Population 7885 (12%)				
46% of population.........................		63	69	51	56
Almost complete	Group III (Wards 12, 13, 14, 15, 16) Population (31%)	23	20	28	26
	Group IV (Wards 19, 20)	12	11	20	18
	Population 14,779 (23%)				
54% of population.........................		35	31	48	44

When we now study the distribution of deaths (about 200), it will be observed that the disparity noted in the relation to the origin of the disease does not hold true, the figures—51% and 48% respectively —are much nearer the ratio of the corresponding population.

The typhoid morbidity, as might be expected, is greatest in the 1st division—being 56% as compared with 44% in the 2nd.

Whenever the apparent relation of the origin of pellagra to poverty is discussed, the argument is usually advanced that the poorer

Chart 12. The relation of the mortality from pellagra (1908-1916) to the method of sewage disposal. The shaded portion represents the part of the city in which sewerage connections are possible.

classes live in the unsewered parts of the city while those economically more fortunate live in the sewered portion, and this in general holds true. In Nashville, however, this condition is to a certain extent (at least in the surveyed area) reversed, inasmuch as a large part of the older portion of the city, now completely sewered, is occupied by an industrial white population and a large negro element (South Nashville), whereas some of the unsewered and newer parts of the city contain in places a very substantial class of white people. From the point of view of the relation of pellagra to sewage-disposal and poverty the situation is therefore of considerable interest in a study of this kind.

If pellagra is due to a dietary deficiency essential to poverty, we should expect that the deaths from pellagra would predominate in the unsewered area, that is, would bear the same relation to the unsewered areas as the origin of pellagra, because, according to this idea, people in the so-called "pellagra class" would not be economically so situated that they could live in the sewered areas.

As a matter of fact, the death rate, as will be observed from the table, is very nearly equal in the two areas under consideration, indicating in a striking manner that the poorer classes are not only equally distributed in the two areas but that many patients who had developed pellagra elsewhere had come to live and die in the so-called better districts.

In the consideration of the equal distribution of deaths from pellagra and the equal distribution of the colored population in the areas under consideration, it becomes apparent that the economic conditions on the whole are also comparable. Hence, the very apparent difference in the numbers of cases of pellagra which have originated in these areas must bear a distinct relation to the method of sewage-disposal, which is, as far as we are able to determine, the only factor of difference in the environment of the people living in the areas under consideration.

The relation of the origin of typhoid fever to the method of sewage-disposal is illustrated in Chart 13. During 1915, 416 cases of typhoid were reported to the city health office, probably the highest morbidity for any of the larger cities in the country.

DETAILED STUDY OF THE EPIDEMIOLOGY OF PELLAGRA

In a discussion of the relation of a constant contact to the development of pellagra in Nashville the fact was brought out that in the case of practically 78.7% of the patients even a cursory history had

Chart 13. The relation of the morbidity from typhoid fever (1914-1915) to the method of sewage disposal. The shaded portion represents the sewered area.

revealed evidence of an association between the development of the
disease and contact with previous cases. We are fully aware that in
a population having a pellagra morbidity of approximately 1%, an
intimate and constant contact is bound to occur among pellagrous
individuals due to purely fortuitous circumstances, not the least among
these being the result of a frequent change of residence from one house
to another; and there is also the undoubted fact that, on the whole,
pellagra is a disease of those economically less fortunate, who, by rea-
son of circumstances, live together in more or less intimate contact.
We nevertheless have been so impressed by the evidence that new
cases develop only in those individuals who somewhere and at some
time have either lived near or have associated intimately with pella-
grins, that we think it might be of interest to record in graphic form
some of the examples that came under observation, the more so since,
with the exception of the work of the Thompson-McFadden commis-
sion, no detailed epidemiologic studies concerning pellagra have been
published in this country.

We do so fully cognizant of the undoubted value and importance
of the work accomplished, which places the etiologic basis of the
disease in purely metabolic disturbances due to the imperfect dietetic
condition of a great mass of people. We believe nevertheless that
thus far the experiments designed to elucidate this point are incon-
clusive and by no means final, that considerable work remains to be
done both in epidemiologic studies and animal experimentation, and
that for the present at least the question of etiology is still an open one.

That pellagra is a disease of certain localities, a disease of "place,"
has been frequently observed in Europe and in this country, and the
situation in Nashville does not differ in this respect from that observed
elsewhere. This grouping in certain well-defined areas in wider dis-
tricts has led in theory to the correlation of the topography and the
geology of the country with the distribution of the disease, giving rise,
among others, to the hypothesis that pellagra is prevalent only in
those regions in which lime salts are deficient in the water and silicates
too abundant. The Italians have emphasized also the fact that even
in the larger pellagra districts, more cases and more severe cases will
originate in certain villages or districts than in others, despite other-
wise comparable economic and hygienic conditions.

The study of the origin and distribution of the disease in Nashville
revealed numerous instances of this situation; almost all cases could

be traced in their origin to some rather well-defined focal area, some of the smaller of which will be described in the following charts.

Chart 14 represents a rather isolated community of white people living in the tip of the 25th ward, an area which contains almost 40 squares. It is surrounded by factories—fertilizer and woodworking factories—and vacant low-lying areas. A railroad branch line forms a partial boundary, dividing the district into 2 parts. The houses are of frame construction, containing 3 or 4 rooms, and are usually separated by a space of 25 or 30 feet. There is no sewerage or city water supply; open and exceedingly unsanitary privies are intermingled with shallow wells. The families frequently move, often to or from North Nashville.

In the area west of the railroad live some 550 people (309 adults, 248 children); the community life centers to some extent about a small park containing several springs, and about a free clinic established by several of the charitable organizations. In this area pellagra has been evident for at least 7 years, some of the earlier patients dating their first symptoms to the spring of 1908 when living in houses along Street C. Inasmuch as no definite addresses could be obtained from these cases they have not been charted, but it will be observed that other cases originated in 1910-11 and in 1912 in the adjoining houses. The greatest number of cases, however, have originated in very recent years, and will be described in detail.

House 1.—A white woman 50 years of age. Stated that she had first had an eruption in 1911, with recrudescence every year following. Address when first attacked not obtained.

House 2.—A white woman, aged 20 years. When the history was obtained, the patient was living in East Nashville, having moved there from House 17. From 1913 to the spring of 1915 she had lived with her father and mother in House 17; her father had pellagra. The eruption had begun in the summer of 1915.

House 3.—A white woman, aged 27 years, who had first had an eruption in 1912 while living in House 42. Her mother and sister-in-law had developed the disease in 1911 or 1912 while living with her in House 42.

House 4.—Two patients had lived here in the spring of 1915. The older, a white woman of 42, had had the disease for several years. While living here the son, aged 20, had developed the disease and after he had moved to House 62 a third case had developed in a daughter, aged 13. Recently another family had moved in. This was the son of the patient in House 35, and brother of the patient in House 7. His 2 children had developed pellagra in 1915 after living in the house.

House 5.—Two cases, both in boys, aged 12 and 13. Had begun in 1915.

House 6.—A woman of 52, living here 7 years, had had pellagra since 1912. In 1913 a woman and her child had come to live with her and both had developed pellagra.

House 7.—A woman of 23 and her 3 children had developed pellagra here in 1915. She was the daughter of an old man living in House 35 who had had pellagra several years. In this house a 15-year-old girl had died in 1911 from pellagra.

House 8.—During the winter of 1914 a woman and her child had lived here who had pellagra; they now lived in House 45. In the family that afterward had moved into House 8 a child had developed pellagra in the summer of 1915. This child formerly had lived at House 9.

House 9.—In this house one patient had been living and another had developed the disease early in the spring of 1914; the occupant at the time of the survey was a woman of about 30, who had had pellagra for several years and had had a typical eruption for at least 5 years. She had moved from House 23 during 1915. Her sister, a woman of 25, living in House 49, had pellagra, and had lived with her at various times. In the spring of 1915 the son, aged 14, had developed pellagra while living here in House 9.

House 10.—A white woman of 48, who had had a typical eruption for several years. She also had diabetes. Her daughter, aged 10, had had a mild eruption in the spring of 1915.

House 11.—A boy of 16, who had had his first eruption when living in House 40, four years before. His aunt and her child, living elsewhere in the city, had died in 1914 from pellagra.

House 12.—A boy in this house, aged 13, had developed pellagra during 1915.

House 13.—A woman of 77 had died here in 1911 from pellagra. No history to be obtained. Two years later a son, aged 44, who had had pellagra for some time, had committed suicide here.

House 14.—A woman of unknown age had died here in 1913 from pellagra; no history to be obtained.

House 15.—A woman of 48 had developed the disease here in 1912. She was living in House 19.

House 16.—In 1912 a white man of 63 years had died in this house from pellagra; no history to be obtained.

House 17.—In this house an old patient had lived for some time, and the daughter had developed pellagra here early in 1915. At the time the history was obtained, the daughter was living at House 2.

House 18.—Two patients had lived here for a short time in 1914; were living in House 24 at the time of the survey.

House 19.—The residence of the pellagrin whose case had originated in House 15.

House 20.—Four cases had developed here in the spring of 1915; these were in 4 girls about 6, 8, 11, and 14 respectively. The family had associated constantly with the family in House 17 during 1914.

House 21.—A white woman of 37, who had had pellagra since 1912, when she lived next door to a case (See Chart 16, House 3).

House 22.—A woman of 43, whose illness had begun 5 years before, when living in House 44. In the spring of 1915 she had had an eruption for the second time. In the following summer her son, aged 17, had had an eruption for the first time.

House 23.—The pellagrin living in House 19 had lived here from 1911 to 1914. The present family had moved in in the spring of 1914, and a case had developed in a girl of 16 years of age.

House 24.—In this house there had been 3 patients of which 2 had recently died. This family had lived for a time in House 26, had then moved to House 9, where another case had developed; then to House 18, and from the spring
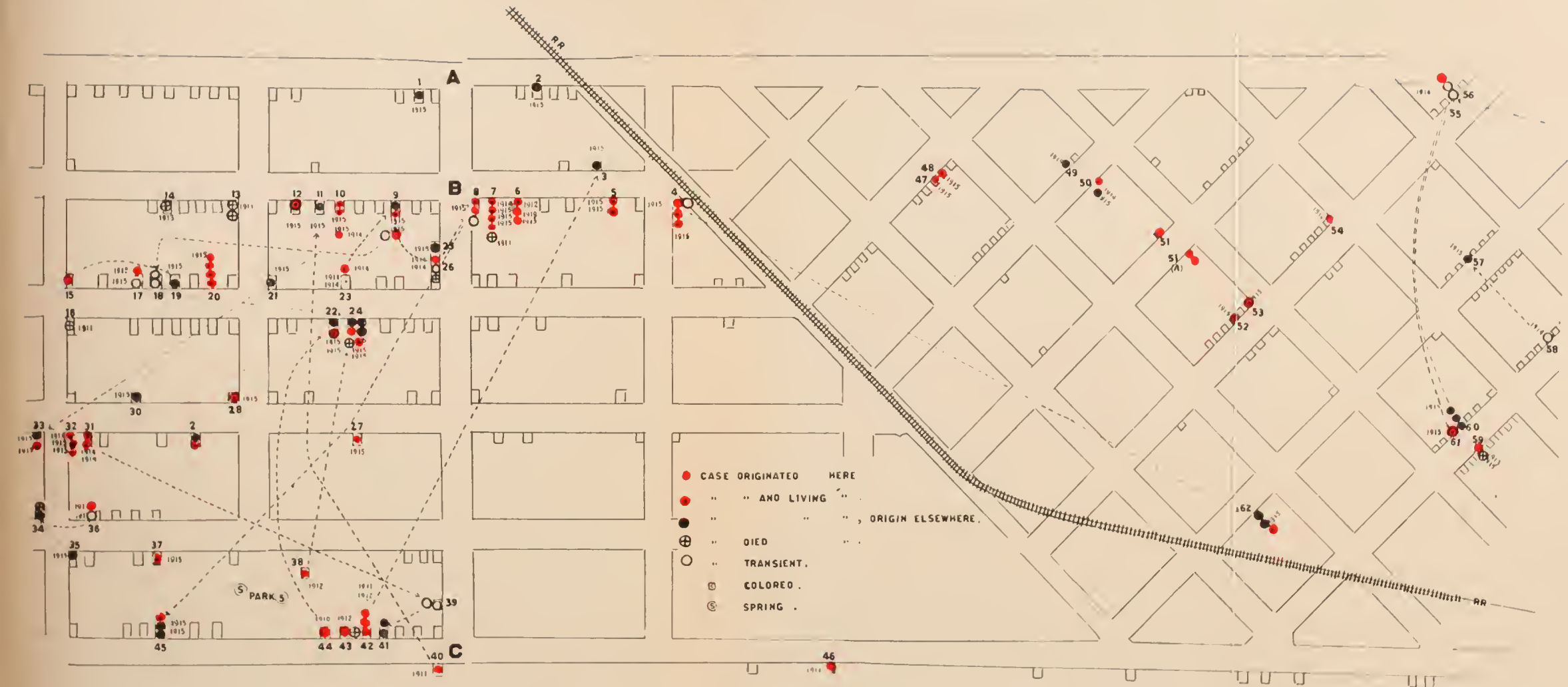
Chart 14. The sequence of cases of pellagra in a part of the 25th Ward. This color key is to be used also for following charts.

of 1915 had lived in House 24. One of the children had died from pellagra here; another had died from tuberculosis but had had a pellagrous eruption in 1914 when living in House 9. Another case, which had originated in House 38 in 1912, was living with this family.

House 25.—A boy of 15, who had had an eruption for the first time in 1914 when living in House 51. He now lived with his grandmother. His youngest sister, aged 3, had recently died from pellagra in House 34.

House 26.—In 1912 a white woman of 56 had died here from pellagra; in 1914 another pellagrous woman had moved in who later had moved to Houses 8 and 45.

House 27.—A white woman of 68, who had developed pellagra during 1915.

House 28.—A man of 57. Had developed pellagra in 1915. This patient had lived in different houses. No history obtained.

House 29.—A woman of 45. Had had pellagra for several years; had formerly lived in South Nashville. Her son, aged 15, had developed a typical eruption in the summer of 1915.

House 30.—An old case of at least 7 years' standing, which had begun in Rutherford county. The woman was 45 years old.

House 31.—Two children, aged 5 and 6 respectively, had developed pellagra when living in this house in 1914; had later moved to Houses 39 and 41.

House 32.—At the same time one case had developed in this adjacent house— this was in a boy of 6. During the summer of 1915, 2 more children in the family had developed typical lesions (aged 9 and 4).

House 33.—A patient living here had developed pellagra in 1912 when living in House 10. Her son, aged 13, had developed pellagra during the spring of 1915.

House 34.—In this house a child of 3 recently had died from pellagra. (A sister was living in House 25.) The history of the family was as follows: Originally had lived in a house not shown on the chart, had moved to House 51, and then the mother had died (from tuberculosis) and the boy who had developed pellagra had moved to the house of his grandmother (House 25). The rest of the family had come to House 36, where they had been joined by an old couple from the country. The old man was a pellagrin. The father of the child had recently developed suspicious symptoms—ptyalism, diarrhea, etc.

House 35.—A man of 66, who had had pellagra for 5 years. It had begun in Hickman county. His daughter and their family lived in House 7; his son in House 4; both families had pellagra.

House 37.—A boy of 13. Symptoms had begun during the summer of 1915.

House 38.—A case had developed here in 1912, and was now in House 24.

House 40.—A case had developed here in 1911; was now in House 11.

House 41.—The patient living here had developed the disease in 1914 in House 31.

House 42.—Three persons had developed pellagra in this house in 1911 and 1912—a woman of 40; her daughter, 19 years old; and her daughter-in-law, 25 years old. The latter now lived in House 3. An infant had died here in 1913 from pellagra.

House 43.—In 1906 a child of 6 years had developed pellagra in the adjoining house. This family now lived elsewhere.

House 44.—The patient who was living in House 22, had developed pellagra here in 1910.

House 45.—A woman of 45 had developed pellagra here 7 or 8 years before when living in North Nashville. This patient in 1914 had lived for a time in House 26, had then moved to House 48. While there, the youngest baby, 18 months old, had developed pellagra. She had then moved to this house and

here her daughter of 12 years, had developed typical symptoms during the summer of 1915.

House 46.—A man of 45 had developed pellagra here in 1911; later had died elsewhere.

House 47.—Girl of 11, living here 3 years, had developed pellagra during the summer of 1915.

House 48.—Boy of 10, living here 5 years, had developed pellagra during the summer of 1915.

House 49.—Woman of 25. The disease had begun 7 years before as a "tetter" on both arms. She had been well for a time but in the summer of 1915 again had had a complete symptom complex. This patient was the sister of a pellagrin in House 9, and had lived with her at various times.

House 50.—In 1914 a case had developed in this house—in a boy of 16. The mother, 45 years of age, had had pellagra for several years, the disease probably having begun in the country. In the house immediately behind 51, had lived a family in 1913 in which one case had originated in 1913, which was now in House 25. In House 51-A, 2 cases had originated during 1914, one in a woman 44 years old, the other in her son, aged 13.

House 52.—A case had developed here in the summer of 1915 in a man of 67. He had been living here for 26 years.

House 53.—At about the same time another case in a woman 80 years old, had developed here, the second door from the previous house.

House 54.—About 3 years before, a woman of 38 had developed pellagra here. Had lived here 7 years before.

Houses 55 and 56.—In House 56, 2 patients had lived during 1914, a woman of 54 and her daughter of 30. The 11-year-old son of the latter woman had developed the disease while in this house. The family then had moved to House 60. In the adjoining house (35) had lived a family who later had moved to House 61. A boy in this family (aged 16) had developed pellagra during 1915.

House 57.—Here there was a woman 45 years of age who had had a typical skin lesion in 1915, and had had a sore mouth for 2 or 3 years. In 1914 she had lived in House 58, and had always lived in the neighborhood.

House 59.—A patient had died here during 1915—a woman 41 years of age, who had suffered from a diarrhea for many years and had had a typical skin lesion for 2 years. Had always lived in this house.

House 60.—The family here had been in House 4 in 1915. One case had originated after the family had moved here.

In a general survey it will be noticed that none of the cases originated, as far as we know, on Street A, despite the fact that economically the people living here are in the same class as the others in the district. They are separated, however, by a rather wide vacant area in the rear of the houses.

The majority of the cases occurred in Street B and the next lower street, and the earliest recorded deaths occurred along these streets (3 in 1911, 1 in 1912, 2 in 1913, 1 in 1915). An old area seems also to have been that along Street C.

Chart 15 illustrates the sequence of cases in a small area in East Nashville. The shaded portion represents the sewered portion. The same color key as in Chart 14 is used in this and following charts.

Chart 15.   The sequence of cases of pellagra in a small area in East Nashville.   Same color key used here as in Chart 14.

House 1.—A patient had died here in 1910—a white man of 25 years—the chief symptoms being diarrhea. No further history was to be obtained.

House 2.—A white woman of 54, now living elsewhere, had lived here during part of the year 1914. She had had all the symptoms of pellagra in 1911, but had since made a complete recovery.

House 3.—A case had developed here in 1914 in a white woman of 33. This patient had come from Sparta, Tenn., about Jan. 1, 1914, had lived here 8 months, and then had moved to another address. She stated that her hands had got "dry" during 1914. At the time that the history was taken she had all the symptoms of pellagra. It is not definitely known whether she had had pellagra before coming here.

House 4.—In the spring of 1915 a patient had lived here. This was a 3-year-old girl, who in the winter of 1914, had lived at the edge of town. This case had developed in House 4, and the patient was now living in House 5. In 1911 a colored woman, aged 33, had died in this house from pellagra. The death certificate stated that death had been due to erysipelas of 4 months' duration.

House 6.—Two cases had developed here in the spring of 1915. The first was in a white woman of 27, who had had a typical attack since March of 1915. Her son, aged 16 months, had had a typical eruption since about the first of August, 1915. The family had lived in this house 3 years.

House 7.—This was a case of pellagra in a white woman, aged 66, who had had her first symptom in 1911, when living in House 8. After that she had been living in the country, but had returned to the city two months since. The disease seemed quiescent. A grandchild living in another part of the city had developed pellagra in the spring of 1915.

House 9.—A white woman of 70, who had lived for many years in another part of the city, had died here, the home of her brother. The wife of her nephew lived in House 10. This patient was 31 years old, and had had pellagra for some years. She associated constantly with the patient in House 6.

House 11.—A colored woman, 29 years old, had died here in 1914. No history could be obtained.

House 12.—A patient had died here in 1913. No history was to be obtained except from the patient in House 7, who it will be noted, had lived near by (House 8) and had associated with her.

House 13.—Three cases had developed here in 1913, and one of them, a child, had died. It is possible that the husband of the family also had had pellagra, but the history is uncertain. The family was living in another part of the city and the mother and daughter were seemingly well. Their neighbor in House 14 had developed pellagra at the same time. The woman had remained mentally affected; the small boy still had occasional attacks of diarrhea. The husband who had deserted the family, was said to have had a bad stomatitis at the time that the wife and the boy had had the symptoms. An infant of 14 months had died in the summer of 1915 of pellagra.

House 15.—In November, 1913, an old man, 74 years of age, had come here from Hillsdale, Simpson county, Ky., with evidences of pellagra, altho a marked eruption had appeared only in 1914. He had lived here with his daughter and had died in June, 1914.

House 16.—Two doors beyond in House 16, a family containing 5 children and 2 adults, had moved in, November, 1914, remaining there until July, 1915. The father, aged 37, had developed pellagra in March, 1915. In June and July, 3 girls, aged 11, 13, and 15 respectively, had developed pellagra. A boy, aged 2, had died in the spring from enteritis and had had a bad stomatitis, but no skin

lesions had been noticed. No physician in attendance. At the time of the survey, the family were living elsewhere.

House 17.—In the spring of 1914 another case had developed here. This was in a white woman of 28, who at the time the history was obtained was in a fair state of health. She had moved to House 18. A daughter, aged 10, was suffering from a stomatitis.

House 19.—A white woman 40 years of age had died during 1914 in this house. No history obtained as to onset.

A rather isolated community is that shown in Chart 16, in which the sequence of cases is rather striking. The district under consideration consists of a square block, divided in the center by a narrow alley.
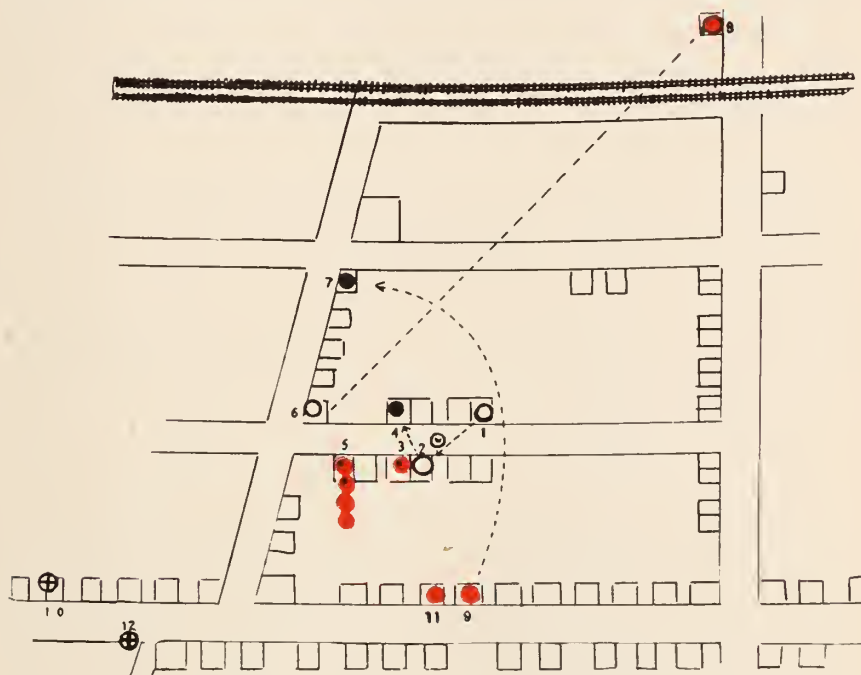
Chart 16. A striking sequence of cases of pellagra. See Chart 14 for the color key.

In this alley-way 5 double tenements are located, and a well (designated W in a small circle) supplies these families and several of the adjoining houses with water. All of these families constantly intermingle, visiting back and forth. There is no sewerage.

House 1.—In 1912 a white woman of 50 years had come here from Lebanon, Tenn. For 2 years previous she had had burning sensations in the stomach, and a tendency toward diarrhea. About 2 years before, she had first had a severe attack of diarrhea, had been very nervous, and had suffered from headaches,

Her mouth had been sore at times and at the time her history was taken she was salivated. The eruption on the hands was typical, but she did not recall the exact time of the onset. We assume that she had the disease when she came to House 1, in 1912. In 1913 she had moved to House 2, and was now living in House 4.

House 3.—In a different part of the city was found a white woman of 37 who dated her first symptoms to 1912, when she was living in this house.

House 5.—In the summer of 1915 a white woman of 45 had developed pellagra in this house. Living as her boarder was a white man of 35, in good health until 1915; he had developed pellagra during the summer of that year. Two other cases had developed in the same house during 1915. One, a woman 28 years of age, the daughter of the woman first mentioned, had developed symptoms of pellagra in the spring. She now lived with her friend a white woman of 32, at a different location. This latter woman had also developed pellagra while living in the house under consideration, but gave a history that her husband had died at an insane asylum and that her mother-in-law, with whom she had been living until she had come to House 4 in the spring, had pellagra.

House 7.—Another patient, a white woman of 38, who had developed pellagra in House 9 about 1 year before, now lived in this house.

House 8.—A white girl of 18 years lived here, who had developed pellagra in March, 1915. She gave a history of having had poor health since childhood, following an attack of typhoid fever. In January she had come from the country, and had then lived for 1 month in House 6.

House 10.—The only other patient in the neighborhood had been one that had died recently in this house. This had been a white woman. No history was to be obtained.

At the county asylum a woman had been admitted in the summer of 1915 suffering from pellagra. From the history obtained the symptoms dated from the summer of 1914, when the patient had lived in House 11. She had later moved to another address.

House 12.—A white man had died here in 1912 from pellagra.

Chart 17 also illustrates the sequence of cases of pellagra.

House 2.—Here there had lived a family with 3 pellagrous children during several months of the year 1914. The family now lived in East Nashville.

House 3.—Here, adjoining House 2, had lived a patient from the fall of 1914 to the summer of 1915. This patient (now in the city hospital for treatment for pellagra) gave the following history. She had had a severe diarrhea for 2 months past. About 4 years before she had had some scaling on both feet and on the inside of the hands, together with some indigestion. Ten years before (she was now 33) she had lived in Sumner county; at that time she had had a severe diarrhea, but gave no history of skin lesion. A number of neighbors had died of "flux" at that time. She now lived on an adjoining street.

House 13.—The patient here (history obtained at the city hospital), a white woman of 55, had had scaling on the back of her hands for several years and sore mouth every summer. Had been salivated 8 years before. The patient had occasional diarrhea and experienced difficulty in walking; was very nervous at times. Since Christmas she had lived in this house. The patient's mother had died from pellagra 8 years before.

House 12.—Here, across the street from House 13, lived a woman aged 27, who gave the following history: Seven years before she had had an eruption on the back of both hands, had been very nervous, and had had a stomatitis.

At the same time her father, mother, and sister had developed similar symptoms. Her friend, working with her as a clerk in a bank, had also had an eruption, diarrhea, and stomatitis. A sister-in-law was found in the survey with early symptoms and it was found that the mother and father of this
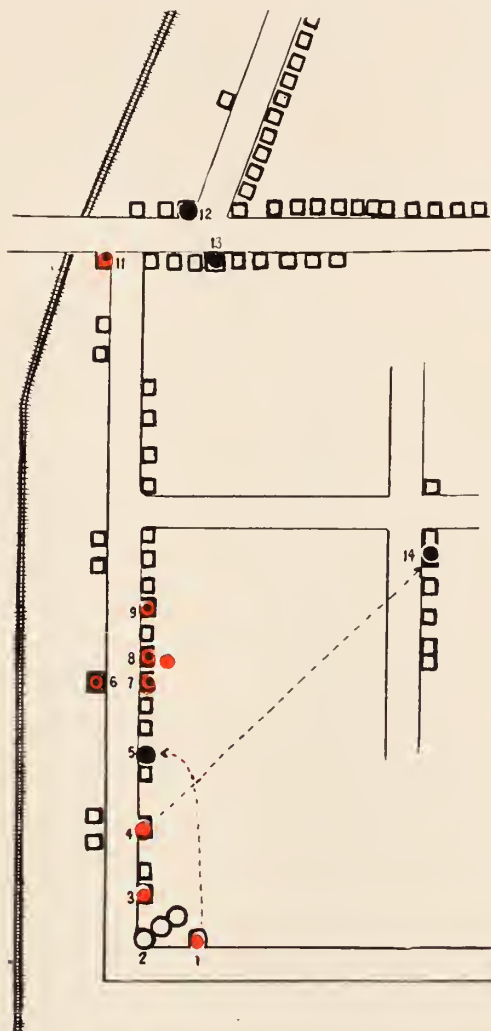
Chart 17. The sequence of cases of pellagra in an unsewered district. See Chart 14 for the color key.

latter woman had died in Nashville in 1910 from pellagra after coming from the country. The patient had lived in this house 1 year

House 11.—Here, diagonally across the street from House 13, a case had developed in 1914 in a white woman of 40; she was now in a fair state of health.

House 6.—A woman of 53, who had lived here for 18 years, stated that for years she had had scaling on the back of both hands in the summer time. For 2 years past she had felt very nervous and had had a stomatitis. She had always had an ample supply of milk, having kept a cow.

House 8.—Here, across the street from House 11, had lived a white woman of 48 for 11 years. In 1911 she had had an attack of diarrhea and stomach trouble; had had "eczema" on the back of both hands, had been nervous and she occasionally had a sore mouth. She was now in a fair state of health. Her daughter, a young woman of 22, living in Jackson county, Tennessee, had had a diarrhea and a skin eruption during the summer of 1915, some stomatitis, but no nervous symptoms. She was visiting her mother at this time. A grandchild (a white girl aged 6) of the older woman, living in an adjoining house (7), had developed pellagra during the summer of 1915.
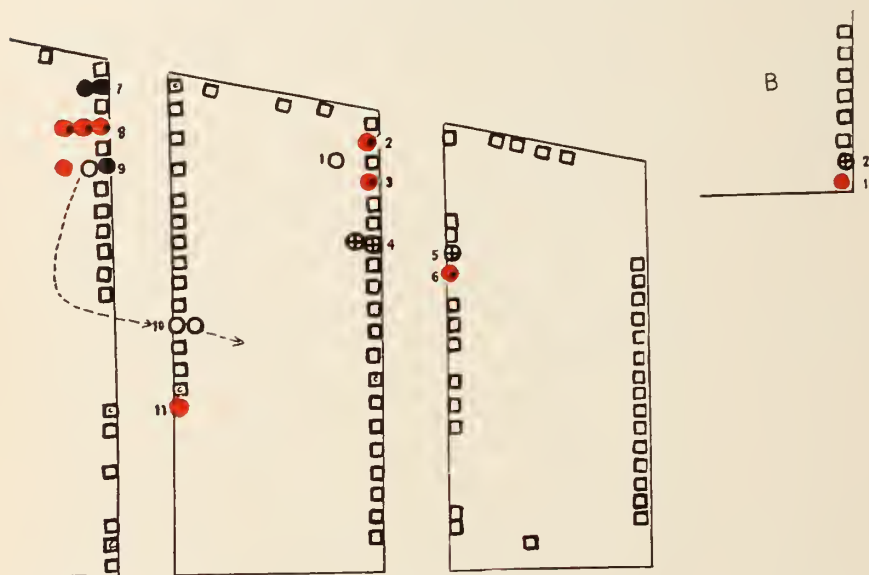
Chart 18. The sequence of cases of pellagra in an unsewered district. See Chart 14 for the color key.

House 9.—Here, a few doors away, another woman had developed skin lesions during this summer. No history was to be obtained from this patient.

House 5.—A white child of 2, who until recently had lived in House 1, had developed pellagra here in the summer of 1915.

House 14.—Here a white woman of 31, lived, who had developed mild skin lesions this same summer. Until 1 month before her history was taken, she had lived in House 4, for 18 months.

.The entire district is unsewered.

Chart 18 illustrates the sequence of cases in another unsewered district.

House 1.—Here, in the rear of House 2, a pellagrin had lived during 1914 —a white woman, 45 years of age, with the following history: The patient first had had symptoms of pellagra 4 years before while living in the country, near Goodlettsville. She had come to the city for a time in 1914, moving into the house noted here, and had remained until spring. She now lived with her daughter, who had recently developed pellagra. The friend of her daughter, a young woman of 24, had also developed pellagra.

House 2.—A girl of 14 lived here, who had moved here in March. At that time she had had an eruption on the back of both hands. The mouth was now typical. Previously this family had lived near some pellagrins on another street.

House 3.—A boy of 7 lived here, who during the summer of 1914 had had an eruption on both hands and feet, together with sore mouth and diarrhea. He was now in good condition.

One of the cases of longest duration had also been in this house—a girl of 16, who had died during the summer of 1915. She had developed symptoms of pellagra 12 years before, as a child, in the spring of 1903, while living at another place in North Nashville. At that time the skin lesions had been variously diagnosed as a "weeping eczema," "tetter," "seven-year itch," etc. The condition had continued, each year becoming worse, and at the last attack the mental condition had become very bad and had remained so until death. The family had lived here 6 years. There was one other child in the family with pellagra. This was a boy 2 years old, who had begun to have the typical eruption in February, 1915, and when examined (September) had had an eruption extending over the arms, shoulders, face, chest, and both legs. The child had been weaned at 1 year, but had had occasional breast feeding until February. The child had died in December, 1915. The mother showed no symptoms of pellagra, nor did the remaining child, a girl of 6.

If we go back for a moment to the former address of this family, where they had lived from 1903 to 1909, we find that according to the city death records a white woman of 70, died in the adjoining house in 1910 with a diagnosis of "dysentery and neurasthenia."

House 5.—Here, directly across the street from House 3, a white woman of unknown age had died in 1913 from pellagra. No information is available about this patient. In the adjoining house (6), lived a white woman of 34, who in the summer of 1915 had had all the symptoms of pellagra. According to the county health officer some of the children in this family had also had a pellagrous eruption. A niece of the patient, living in another part of the city, had pellagra.

House 7.—Two patients lived here. The older of these, a white woman of 67, a widow, had had pellagra for several years; she had come to Nashville 10 years before from Smith county, where her husband had died with symptoms which, as described, were pellagrous. She had lived here for a short time (4 months). Her daughter-in-law, a young woman of 25, had had pellagra for 3 or 4 years. She had had no attack during 1914, but had had an eruption in the spring of 1915. She had lived here 3 months, and before, in Smith county, where a neighbor, a cousin, had died from pellagra.

House 8.—A white woman of 30, living here, stated that in the spring of 1915 she had had a scaling eruption on the back of both hands, together with a severe diarrhea. She was very nervous. Had had no trouble like this before. Her two children, a boy of 8 and a girl of 11, had had mild symptoms in the spring of 1915.

House 9.—A case had originated here in the spring of 1915—in a girl of 3 years. The family had contained one other pellagrin. In the summer the family had lived for a short time in House 10, but had since moved, and were not found in the survey. In the same house there lived a man of 66, who had had mild symptoms of pellagra for several years, originating in DeKalb county. At a short distance was found a very severe case in a young negress, who in 1914 had lived for a time at House 11. She was a cook and had during recent times lived in various parts of the city.

Chart 19 illustrates the points of origin of several cases which have occurred during the past few years. At the time of the survey, only one of these was living on this street. She gave the following history:

House 1.—A white woman, 41 years of age, who had had her first eruption on both arms and a sore mouth in 1913. In 1914 she had had no eruption but had had a second eruption in 1915. She had lived in this house 8 years. A relation (Case 443, male, white, 59 years) had had pellagra for several years. He formerly had lived in the vicinity and now lived in South Nashville.

House 2.—Here, directly across the street from House 1, had been a case until 1913. The patient had then moved to House 9. She was a white woman, aged 36. She stated that she had had an eruption on both ankles in January, 1913, followed in March by sore throat, diarrhea, and indigestion. The eruption on the hands had appeared in April, 1913. She was in fair condition now, except for headache and rather bad memory.

House 3.—A white woman had died of pellagra here in 1912. No history could be obtained about this patient. In 1914 a white woman of 27, had developed pellagra in this house after living here 1 year. She stated that her husband's grandmother, an old pellagrin, had lived in the house with her and during this time she had had her first eruption. She had later returned to the country. This family was now in House 4.

House 7.—A case had developed here in 1913, during the spring, in a white woman, aged 42. She had been a friend and a frequent visitor of the patient living in House 9. In the autumn of 1915, this patient had moved to House 8 and had died there.

House 5.—A patient had lived here in 1913, had moved during the latter part of the year to House 6, and there had died during the same year. A sister-in-law (Case 831) living in North Nashville, stated that she had had a skin eruption and sore mouth for several years. One other case had died in the neighborhood, in House 10 (1914). No details were to be obtained about this case, nor about a patient who had died in 1911 in House 11.

The sewered area is shaded.
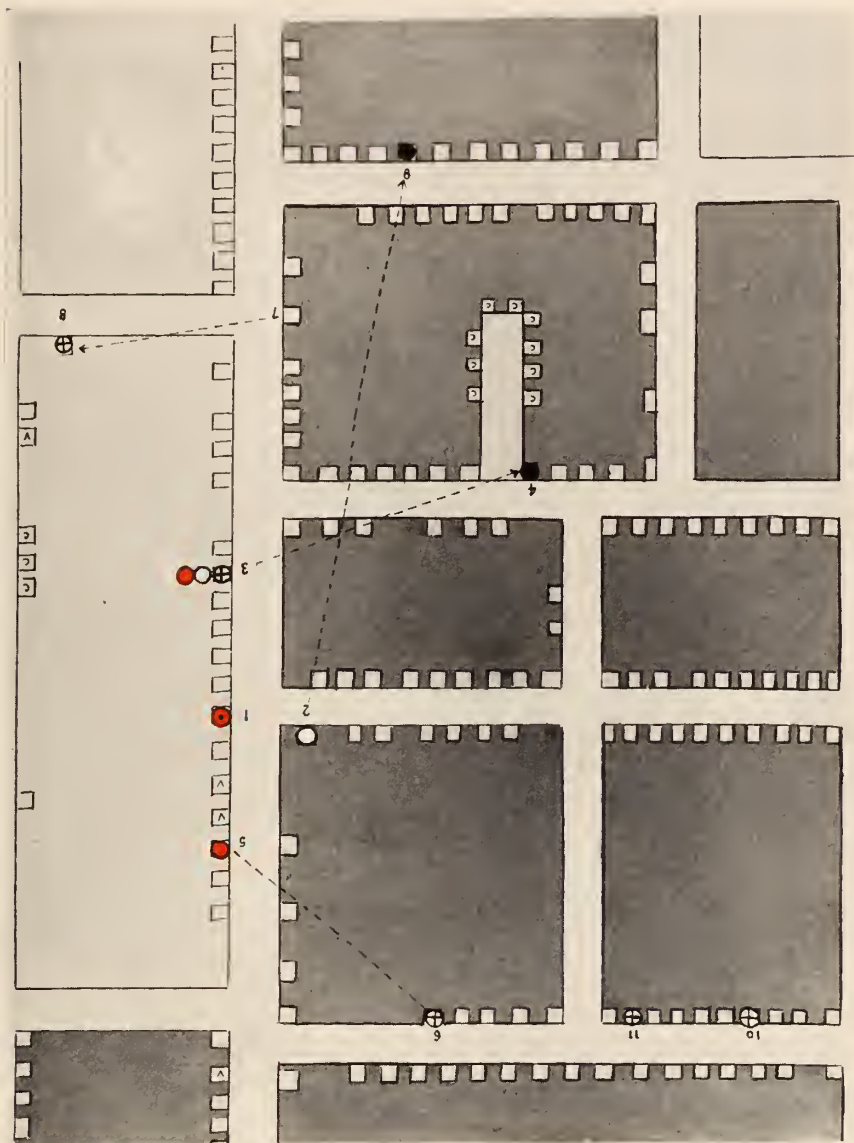
Chart 19. The points of origin of several cases of pellagra. The same color key is used here as in Chart 14.

In Charts 20 and 21 two families are shown, both of which are in moderately good circumstances, and both of which have been under expert medical care and careful instructions as to diet for several years. There is no sewerage in the areas in which they live.

Chart 20.—In 1911 a young woman, 20 years of age, came from Huntsville, Alabama, where she had married a few years previously, to live in Nashville. While in Alabama she had nursed several friends who had pellagra. No members of her family had the disease at that time. She first lived in the house of her father-in-law (House 1) where she developed the disease shortly after her arrival. She remained here until the spring of 1914. During this time her sister came from Alabama, lived in the house for a time, and developed the disease there in 1914. She later recovered and now is living in House 2. In this house a third sister, now 19 years of age, lived with the second sister and while here developed symptoms of pellagra in the spring of 1915. She now lives in House 3.

In the spring of 1914 the original case moved with the father-in-law's family to the next street (House 4). Here both the father-in-law, a man of about 70 years, and the mother-in-law, aged 66, developed the disease. Later in the summer of 1914 the family moved to the next street, House 5. Here the father-in-law died, and his 2 younger children, aged 5 and 7 respectively, developed the disease. The mother-in-law was taken to the county asylum, where she died in 1914. The family has since moved from this house.

Chart 21.—A white woman 34 years of age, was taken ill in 1910 with a serious attack of pellagra. The family lived in House 1, having moved in from the country 2 years previously. The nearest neighbor on the farm in the country with whom the patient had frequently associated had been Mrs. E. R., who died 2 years ago in Franklin, Tenn., from pellagra, altho at the time of association the patient had not been aware that she had pellagra. The oldest son, aged 5, also developed the disease at the same time that his mother had. They were nursed by a woman living in House 2 who has remained well. The family remained in House 1 for another 3 years, then moved to House 3 (1914), where the patient had another severe attack, almost dying. At the same time the younger son developed a mild case of pellagra. Immediately across the street, in House 4, lived the sister-in-law. In this family the oldest son, aged 9, developed typical skin lesions in the summer of 1914, altho, as the mother stated, he might have had some eruption a year or so before. In the spring of 1915 the sister-in-law developed typical skin lesions and had a severe stomatitis.

After the original patient had recovered in the fall of 1914, the family again moved, this time to House 5, where they have lived since September, 1915. In House 6 adjoining, a mother and her 3 children all developed mild lesions in the spring of 1915. They have recently moved. A brother-in-law of the original patient, together with his child, have recently developed pellagra (examined by the county health officer) but the present address is unknown.

There is only 1 other case in the immediate neighborhood—a white woman, aged 33, who has been "nervous" since 1914 and had a "sunburn" on both arms

Chart 20. The sequence of cases of pellagra in a family group in an unsewered area.
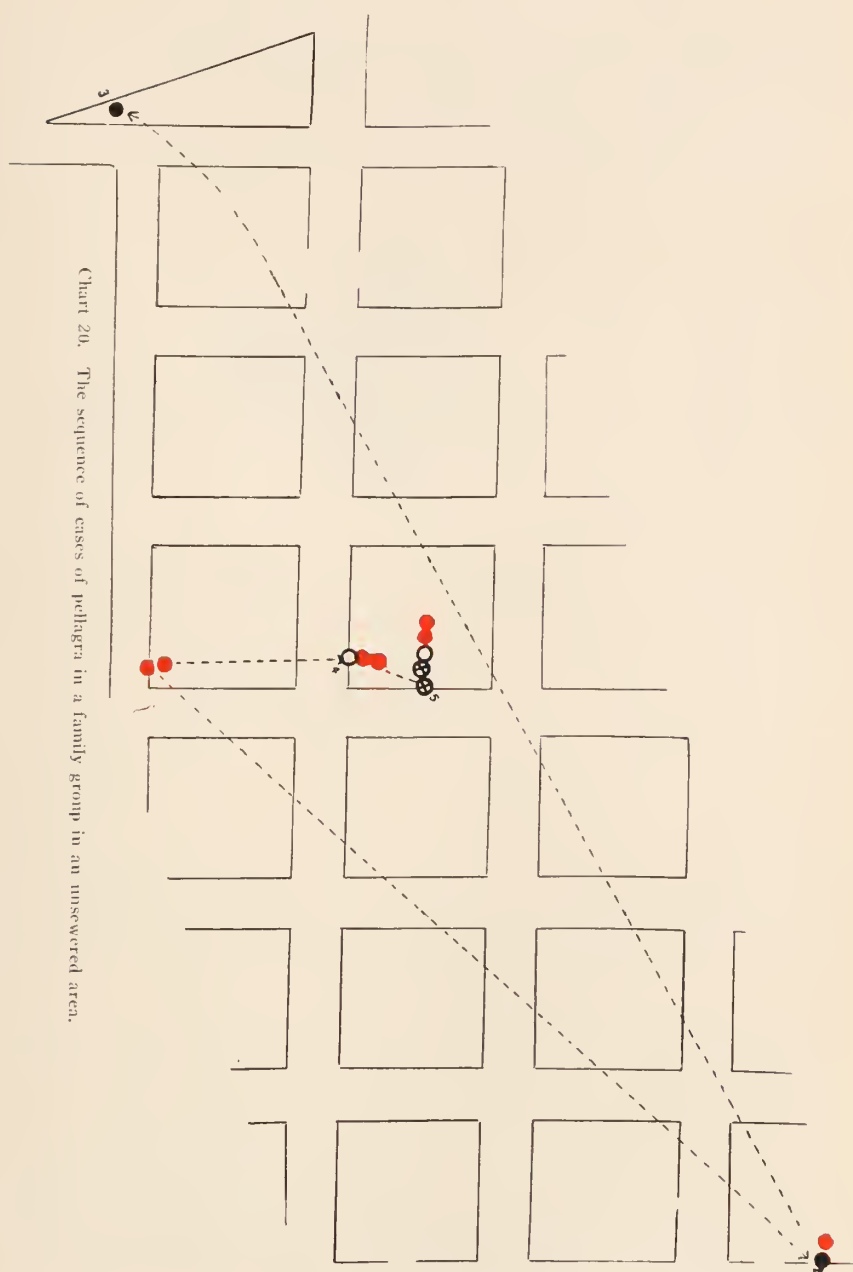
for the first time in the spring of 1915. Two years ago there lived for 2 weeks in her home a woman who was ill, and who was taken north, after a diagnosis of pellagra had been made. It is believed that she has recovered.

In 1914 a patient, it is said, lived in House 8 for several weeks.

In 1911 a case of pellagra originated and terminated fatally in House 7.

The wells which supply the families are indicated by small circles.
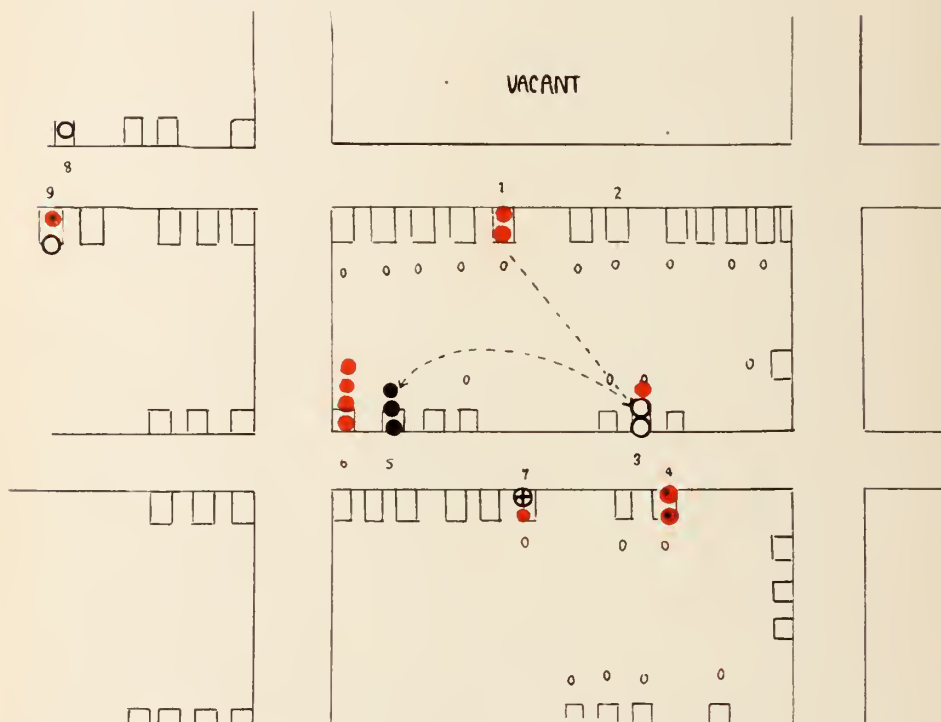
Chart 21.   The sequence of cases of pellagra in a family group in an unsewered area.

RURAL SURVEY

During the summer (July and August) of 1914 a careful sanitary survey of a rural district adjacent to Nashville was made under the direction of the county health officer by Dr. W. B. Garner and Dr. T. V. Woodring.

In this surveyed area, which lies north of Nashville and includes all of the 10th and part of the 14th county districts (Chart 22), about 385 houses were visited and approximately 1725 individuals examined for pellagra. In this number 1592 were whites and 131 negroes; the average number of inhabitants to each house was 4.5, the same ratio that was found for Nashville.

The surveyed area is representative of the country surrounding Nashville. Parts of the district are comparatively fertile; others, sparsely inhabited portions, are rather barren. The crops consist largely of small grains, tobacco, and corn; stock and dairy interests are negligible; there are no manufacturing plants of any kind in the region.

Chart 22.   Map of Davidson county showing extent of the rural survey.

The sanitary conditions are probably better than in the average southern rural community, but of the total white population about 85% live in houses where absolutely no provision has been made for the disposal of human excreta. Of the total negro population of this area (131), only 3 families live in houses with access to privies.

In the surveyed area we found 39 cases of pellagra, all among the white race, representing about 2.5% of the total white population. These 39 cases occurred in 29 houses, 13 of which had no provision for the disposal of excreta, the rest having open privies. The total number of people living in these houses was 125. Of the 39 cases, 26 occurred in males and 13 in females. The age distribution is shown in Table 2.

TABLE 2

DISTRIBUTION OF CASES OF PELLAGRA ACCORDING TO SEX AND AGE

Ages.....	5 to 10	11 to 15	16 to 20	21 to 25	26 to 30	31 to 35	36 to 40	41 to 45	46 to 50	51 to 55	56 to 60	61 to 66	67 to 70	71 to 75	76 to 80
Males....	2	1	2	1	0	1	4	5	2	1	2	2	1	0	2
Females..	1	1	1	0	1	4	2	2	0	0	0	0	0	1	3

10TH DISTRICT

Chart 23.   The grouping of pellagrous houses in the surveyed area shown in Chart 22.

It will be noted that the ratio of males to females is quite the reverse of that found in the city survey.

In Chart 23 the pellagrous houses have been indicated on a map of the surveyed area. The invariable grouping of 2 or 3 adjacent houses is evident.

The majority of the observed cases are of recent origin, dating back not more than 3 or 4 years at the outside. As far as we are able to determine there has been no alteration in the diet during this time, altho the diet is no doubt far from ideal. Carbohydrates and fats predominate; during the summer considerable quantities of fruits and leguminous vegetables are added. In some respects the diet of these rural families has deteriorated during the past 20 years on account of the absence of small game, due to the exhaustion of the timber supply, which formerly offered a ready means of augmenting the family budget; and on account of the unfortunate substitution of canned goods in the dietary in place of vegetables produced on the place. At present the diet consists largely of fat meat, ham, and occasional fowl, potatoes, beans, turnip greens, fresh onions, and fresh fruit. About one-half of the families use corn products; the other half, wheat flour for bread and biscuits. Most of the families have a cow, so that considerable milk is consumed, but very few eggs are used. In quantity this diet is possibly superior to that of the city worker, but it is somewhat less varied and undoubtedly contains less fresh meat.

DISCUSSION

The data gathered during the progress of the survey indicate that pellagra has been present in this locality for a longer time than is generally believed. If the information obtained from one patient is correct, she had her first attack in 1891, but the results of our work lead us to believe that most of the cases developed subsequent to 1907, and a great majority within the past 2 years.

It has been said that pellagra was just as prevalent in the latter part of the nineteenth century and earlier part of the twentieth century as it is as present, but that it was not recognized as an entity and that the majority of cases have since died.

This assertion is, we believe, disproved by the evidence submitted by Dr. West and Dr. Tucker. Their intimate knowledge of the health conditions of this locality obtained in their capacity as public health officials during the past 10 years, gives them the right to speak

with authority on this phase of the subject. That it was not prevalent in 1909 is shown by the results of the investigation of several of the local state and county institutions by Dr. Lavinder of the U. S. Public Health Service. Dr. Lavinder at that time expressed the opinion that pellagra would probably be found in most, if not all, of the charitable institutions in this locality, but after a careful examination he was unable to find a single case in any of the institutions examined. This is important, because as a result of a very cursory physical examination of the inmates of one of the same insane asylums we found nearly 30 cases—approximately 10% of the inmates. Special significance is attached to these findings as a certain number of those afflicted with pellagra become insane and must be confined to asylums; therefore, Dr. Lavinder's failure to find a single case in 1909 and our results in 1915, may be taken to indicate that the disease has become much more prevalent.

The area of the city surveyed contains about half of the population of the city, or approximately 65,000 people. It includes sewered and unsewered areas, and people whose economic condition ranges from to the worst to the best.

In the course of the work it became evident that the disease is not distributed uniformly throughout the population, but that it is confined more or less to certain areas. This localization cannot be satisfactorily explained by the economic condition of the people in these particular areas, as those in similar circumstances living in houses separated by open ground, but several hundred yards distant, are not affected.

The most striking fact brought out by the survey is the relation the disease bears to sewered districts. Chart 11 (p. 537), which shows the origin of the cases, demonstrates this most conclusively. This map is incorrect in that it indicates that many of the older cases originated in sewered districts, when, as a matter of fact, the districts were not sewered at the time these cases developed. It has been interesting to note how the number of cases originating in a given area rapidly decreased subsequent to the installation of sewers. Moreover, many of the cases now living in sewered areas developed the disease elsewhere. Our observation that the disease is much more frequent in unsewered districts confirms that of the Thompson-McFadden commission.

In criticism of the report of the Thompson-McFadden commission it has been said that the people living in houses connected with sewers

were better off economically than those living in houses not connected with sewers, and that this explained the difference in morbidity; the supposition being that the rent of the former was higher than that of the latter. This criticism, however, certainly does not hold true for the surveyed area in Nashville, where there are what are termed "alley sewers." The privies in these instances are situated in the rear of the lot close to the alley along which the sewer runs, and each contains the usual "flush basin." Inquiries were made of the local real-estate agents who had charge of these houses, as to rents, etc., and it was found that there was no difference in the rent of houses of the same size, regardless of their sewer connections. At least 60% of the houses in sewered districts had sewer connections of this character, and as the rent is the same, it can hardly be said that these people are better situated economically. It is also the type of sewer connection, the installation of which apparently has been instrumental in materially lowering the morbidity in certain localities.

Little need be said about the age of the patients. Children do not appear to be as susceptible as adults, and this is particularly true of children during the second decade of life. After the second decade women are affected from 3 to 4 times more frequently than men, tho from 50 years on both sexes appear to be equally susceptible. These results conform closely with reports made by others, both in this country and abroad, and particularly with the statistics published by the Thompson-McFadden pellagra commission. It is interesting to note that these proportions are exactly reversed for the 10th sanitary district, where it was found that more men than women were affected.

Of the total number of cases of whom we could obtain any record, 785 were white people and 235 were negroes, a proportion of 3 to 1. These figures indicate that negroes are just as susceptible to the disease as white people, as the proportion of colored to white people is the same for the population of the city. However, this does not hold true for our survey, as we found that 1 in 106 white people were affected and 1 in 185 colored people. The data obtained during the survey make it appear that the mortality is higher among negroes than among white people. This, if true, would explain the disparity in figures just noted.

The figures given in some reports indicating that negroes are more susceptible than whites can probably be interpreted as more relative than actual, the disproportion being due possibly to the fact that there are more negroes than whites living in these localities.

The majority of the patients had their attacks during the spring and summer months, the white people most frequently during the spring, and the colored people during the summer months. Many of the patients complained of having suffered for some time previous to the acute attack with symptoms referable to the gastro-intestinal tract. Whether or not these symptoms may be considered as belonging to the early stages of the disease, or as merely coincidental, remains to be determined. Nervousness is another symptom which may precede the attack for weeks and sometimes for months.

Practically half of the patients whose histories we could obtain first developed the disease in 1915, while 14% had their first attack in 1914. Thus it will be seen that nearly 65% of the cases have developed within the past 2 years. It is possible that these figures do not give the real facts, and that the disease has been more prevalent in the past than they indicate. If this is true, the mortality in the past must have been greater than it is at present. However, the reports of Dr. Tucker and Dr. West and information obtained during the survey make us believe that these figures represent the real conditions as they exist in Nashville.

We are unable to form even an approximate idea of the mortality, because of the fact that we do not know how many cases have really developed during the past 2 years. That we have not found all the cases in the surveyed area we feel certain, and until such exact information is obtained, it is useless to hazard a guess as to the proportion dying.

It is evident that the inhabitants of the South consume excessive amounts of carbohydrates and fats. Corn in the form of corn-bread and grits has been used a great deal, but it is being gradually supplanted to such a degree in many pellagrous families by wheat flour that it can hardly be considered as an etiologic factor.

Proteins do not occupy the important place in the diet that they do in the northern sections of the country. However, if the data which we have collected are correct, the amount consumed by 68% of our cases is considerably more than sufficient to sustain them. This view is based on the experiments of Chittenden,[6] and the observations of McCay,[8] both of whom found that individuals getting 40 gm. of protein a day do not develop pellagra. It is very probable that a low protein intake does lower resistance; other faults in diet would probably have the same effect. The criticism may be made that the patients and their friends misled us as to the amount of proteins consumed,

but even if we accept the statements of only half the 60%, we still have 30% of the patients who have received much more than 40 gm. of protein a day. Experiments are now being conducted which we hope will give us more specific data on this phase of the subject.

The valuable work done by Goldberger and his associates[3] shows that a properly balanced diet is one of the most efficient means of preventing the development of pellagra. However, it must not be forgotten that poorly nourished individuals are prone to contract many diseases. Centainly our results indicate that other conditions must also be considered in determining the etiologic factor.

Funk[12] has advanced the theory that pellagra is due to a deficiency in vitamins. He states that the disease is caused by the excessive use of devitamized corn, and that it may be prevented by the addition of vitamin-containing substances, such as potatoes, milk, butter, fruit, etc. The people in this section of the country, however, eat a great deal of potatoes, fruits, and other green foods, both cooked and raw, during the spring and summer. It seems strange, if this theory is correct, that pellagra should be rare in the winter when green foods are scarce, and so frequent in the spring and summer when green foods and fruits are plentiful and cheap.

One of the most striking facts brought out in the investigation is the close association of the pellagrous areas with the unsewered districts. A number of pellagrins were found in houses with sewer connections, but the majority of these had either recently moved from unsewered districts, were living in houses adjoining such districts, or were in the habit of visiting friends and relatives who had the disease in other parts of the city.

The sanitary conditions present in these localities are frequently of the worst possible character with regard to sewage-disposal. Many of the privies have vaults, but these are rarely cleaned and are almost never enclosed so as to prevent the entrance of flies. In many instances, however, there are no vaults, and here but little pretense is made to do anything with the excreta, which are usually covered with flies, the chickens and hogs acting as the scavengers. A few of the privies were properly cared for, but these cases were in the minority.

Screening was absent from nearly all the houses and where present, was usually full of holes.

[12] München. med. Wchnschr., 1914, 61, p. 698.

Information obtained from the real-estate men shows that rents for these small houses are the same in a given locality, regardless of whether the houses are connected with sewers or not. This indicates that those living in the houses having sewer connections need not be of different social strata, a view confirmed by those making the survey.

In view, then, of the fact that no other difference could be found between the people in the unsewered and those in the sewered areas to explain the greater prevalence of the disease among the former, it appears that the lack of proper sewage-disposal must play an important part in the development of pellagrous centers. It is also very suggestive that the typhoid incidence, with one exception, conforms closely with that of pellagra.

We were unable to find that water bears any relation to the disease. City water, and water from wells, springs, and cisterns was used indiscriminately, without any apparent influence on the incidence of the disease. The well and spring water is "hard water," because of the presence of lime salts; this would tend to disprove the theory advanced by Allessandrini and Scali[11] that the disease is due to the presence of colloidal silicates, which are removed by adding lime.

Another striking fact brought out in the survey is that 78.8% of the patients had been in intimate contact with other pellagrins before they themselves had developed their first attack. Of this series 33⅓% of the pellagrins developed the disease in houses in which there were other patients; 18.7%, where there were cases in the adjoining houses; 19.3%, where there were patients in the same block with whom they associated; and 7% visited patients elsewhere or were associated with them in their daily occupations.

These figures may be interpreted in two ways: first, that pellagra develops only in those who have been exposed to the disease, and second, that the high percentage of contacts is purely fortuitous, without significance as far as the etiology of the disease is concerned. We are not in a position yet to say which of these two possibilities is the correct one, and we believe that it would be fruitless to attempt any discussion until more proof has been obtained.

In its distribution the disease bears no definite relation to the density of population. It was formerly thought that pellagra was a disease confined more or less to rural districts, but our experience shows that it also occurs in thickly settled communities.

The present report on the epidemiology of pellagra will be followed by another dealing more specifically with certain phases of the etiology of the disease.

CONCLUSIONS

A few cases of pellagra were probably present in Nashville in 1891, but the disease did not become prevalent until 1908. About 65% of the cases found in the survey developed during 1914 and 1915. Approximately 89% of the pellagrins developed the disease while residing in houses which were not connected with sewers, and 95% of these had unscreened vault or surface privies in close proximity to the house. Only 2% of the houses were screened. The typhoid incidence corresponds with that noted for pellagra.

More than 78% of the patients had associated with other patients before they developed the disease. Thus, 33% of the pellagrins developed the disease in houses which contained other cases, while in most of the remaining instances there were patients in the adjoining houses or within the same block with whom they constantly came in contact.

The water used was derived from various sources; it can therefore hardly be considered an important factor in the development of the disease.

White and colored races appear to be equally susceptible. More white people than colored people develop the disease in the spring. The reverse holds true for the summer months. The mortality appears to be greater in the negro race. Relatively few cases were found among negro children.

Individuals from 10 to 20 years of age are less susceptible than at any other period of life. Women 30 to 50 years of age are from 3 to 4 times more susceptible than men. Over 50 years and under 10 years, both sexes are equally susceptible. In the rural district surveyed, more men than women were affected.

The diet contains an excess of carbohydrates, but 68.8% of the patients gave histories indicating that they were consuming proteins in considerable excess of 40 gm. a day, an amount which has been shown to be sufficient to sustain life without the development of pellagra.

IMMUNOLOGIC STUDIES ON HODGKIN'S DISEASE [*]

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In 1910 Fraenkel and Much[1] demonstrated a peculiar granular gram-positive bacillus in stained sections of lymph glands from Hodgkin's disease. In 1912 he[2] announced the demonstration of this organism in over 30 cases. All efforts at cultivating the organism, however, had been futile.

Negri and Mieremet[3] were the first to announce the isolation of the organism. Because of its peculiar morphology they classified it as a corynebacterium, and because of its association with Hodgkin's disease, as Corynebacterium granulomatis maligni. An attempt in two cases to show the presence of complement-fixation antibodies and agglutinins failed. The results of inoculation of the organism into animals were indefinite.

In 1913 Bunting and Yates[4] described the isolation of a pleomorphic diphtheroid organism from the lymph glands in 4 of 7 cases of Hodgkins' disease. In 1914[5] Bunting concluded that "Hodgkin's disease is an infectious disease due to a diphtheroid organism, the Bacterium hodgkini." Following their cultivation of the organism, they[1] inoculated a rhesus monkey repeatedly with 24-hour-old cultures of this bacillus. After 3 months, tissues excised from some of the enlarged lymph glands revealed histologic changes similar to those of glands early in Hodgkin's disease; namely, a chronic lymphadenitis with typical proliferation of endothelial cells, a beginning proliferation of the stroma, and a well-marked eosinophilic infiltration with a periglandular sclerosis.

Subsequently, Billings and Rosenow[6] confirmed these cultural results and isolated the organism from the blood in a few febrile cases. They also added the use of autogenous vaccine to the therapeutic

[1] Ztschr. f. Hyg. u. Infektionskrankh., 1910, 67, p. 159.
[2] Deutsch. med. Wchnschr., 1912, 14, p. 637.
[3] Centralbl. f. Bakteriol, I, O., 1913, 68, p. 292.
[4] Arch. Int. Med., 1913, 12, p. 236. Jour. Am. Med. Assn., 1913, 61, p. 1803.
[5] Bull. Johns Hopkins Hosp., 1914, 25, p. 177.
[6] Jour. Am. Med. Assn., 1913, 61, p. 2122.

measures employed in the treatment of this disease. This led directly to the question of treatment with an immune serum.

At Dr. Billings' suggestion I undertook to ascertain whether an immune serum could be produced which would have either palliative or curative action in this disease. In April, 1914, immunization of a horse was commenced with cultures of organisms from the lymph glands in Hodgkin's disease. The Memorial Institute for Infectious Diseases afforded the facilities for the production of the immune serum, and Dr. P. G. Heinemann gave advice and assistance in the concentration of the serum.

IMMUNIZATION OF A HORSE AGAINST THE DIPHTHEROID BACILLUS OF HODGKIN'S DISEASE

Sixteen strains of diphtheroid organisms obtained from cases of Hodgkin's disease by Dr. E. C. Rosenow and Dr. Garde, were employed in the inoculation of the horse. Killed bacilli equivalent in amount to the 24-hour growths on 2 Loeffler's serum slants were injected subcutaneously. (The bacilli had been killed by heating for 1 hour at 60 C.) This dose produced a mild reaction in the horse, the temperature running up to 101 F. on the evening of the day following the vaccination. A 2nd dose of 10 slants of killed bacilli was given subcutaneously a week later, followed in a week by a 3rd dose consisting of 15 slants. Mild reactions were produced by these injections. A 4th dose of 20 c.c. of dead organisms—approximately 6 billions of bacteria to each cubic centimeter—was injected intravenously in the 4th week.

The needle of the syringe had been withdrawn from the vein but a few seconds when the horse began snorting, pawing, and throwing back its head, making at the same time several attempts to jump over the stall. Collapse followed, with slow difficult respiration, trembling of the legs with inability to stand on them, drooping of the head, and marked general weakness, the animal being held up by a supporting harness. At the end of 3 minutes the horse was trembling violently and drops of sweat had appeared over the entire body. Gradually strength returned, so that the animal could stand alone, and the shaking tremors diminished altho sweating increased, the sweat dropping continuously from the belly. The horse, still weak, was returned to the barn after 30 minutes. That evening its temperature was 103 F., a rise of 4.4 degrees since morning. The next day the animal was much stronger, the temperature (Chart 1) being practically normal. I have discussed this matter in detail as it is undoubtedly an anaphy-
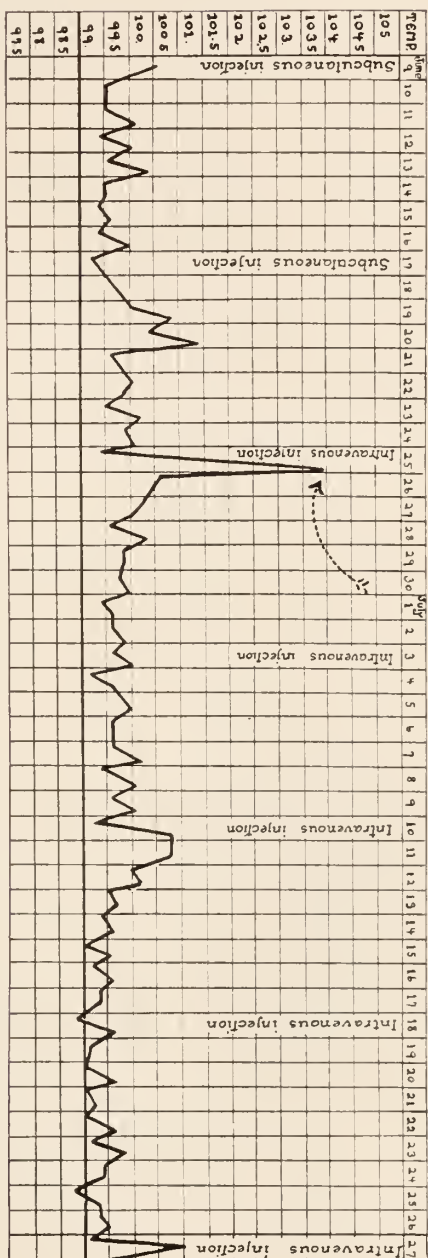
TEMPERATURE CURVE OF HORSE SHOWING RISE AFTER FIRST INTRAVENOUS INJECTION OF DIPHTHEROID BACILLI

CHART 1

lactic reaction in a horse. The rapidity of onset, respiratory symptoms, collapse, and recovery are similar to those described by Nichol[7] and others as allergic reactions in human cases. I find in the literature no similar case of anaphylaxis in a horse.

Subsequent doses of 20 and 25 c.c. of dead bacilli and of from 20 to 30 c.c. of living organisms produced at most very mild reactions. After 3 intravenous injections of dead bacilli, live organisms were employed. The routine dose finally adopted was 30 c.c. of the mixed living cultures. These organisms were grown for from 24 to 72 hours on Loeffler's serum agar, were then suspended in sterile salt solution, and centrifugated to throw down the coarse particles. Each cubic centimeter averaged from 3 to 4 billions of live bacilli. Injections were made intravenously from every 7 to every 10 days.

Except that described, the reactions were mild, consisting of a rise in temperature of from 0.5 degree to 2 degrees. The effects produced by both dead and living bacilli were the same. Chart 1 illustrates the temperature curve of the horse, showing the high rise in temperature after the 1st intravenous dose and the slight rise following the subsequent injections. In a previous paper by the author,[8] describing the production of antistreptococcic serum in horses, it was shown that, altho relatively small doses of killed streptococci were injected subcutaneously, the temperature ascended from 1 to 5 degrees after each injection. One is therefore led to conclude that the diphtheroid bacillus of Hodgkin's disease is of a low degree of virulence. Experiments on other species of animals give further support to this observation.

When the serum showed a high degree of potency, the horse was bled, 2 gallons being obtained each time. Bleedings were made every 3 or 4 weeks. The major portion of the blood serum was then refined by a modification of Banzhaf's method, which is similar to that employed in the refining of diphtheria antitoxin. The plasma is diluted with one-half its volume of water and enough saturated ammonium-sulfate solution added to make a saturation of 30%. It is then gently heated for 2 hours, being brought gradually to an end temperature of 60 C. While the precipitate is warm, it is filtered off rapidly and extracted with saturated NaCl solution. "The filtrate is measured and enough saturated ammonium-sulfate solution added to bring the saturation to 54%. The precipitate is then filtered off, pressed out to remove as much liquid as possible, and finally dialyzed for from 7 to 9 days, or until practically all the ammonium sulfate is removed.

"The salt-solution extract of the first precipitate is thrown down with acetic acid, the precipitate pressed out, and the NaCl and ammonium sulfate dialyzed out. This precipitate must be neutralized with sodium carbonate. The dialyzed globulin solutions are sterilized by filtration through Berkefeld filters. Tricresol (0.3%) is used as a preservative.["][9]

Various tests for determining the potency, or the antibody content, of immune sera are in use, each one depending on the specific antibody formed in greatest proportion, and usually, therefore, only one test is applied in a given instance. For example, in the determination of

[7] Jour. Am. Med. Assn., 1914, 63, p. 2225.

[8] Jour. Infect. Dis., 1914, 15, p. 215.

[9] Am. Jour. Pub. Health, 1912, 2, p. 43.

the value of antidiphtheric or antitetanic serum the antitoxin content is found; in the case of antityphoid serum the agglutinin value; in that of antistreptococcic serum the opsonic value; and in that of the antiserum for Rocky Mountain spotted fever[10] the potency titer is the amount of immune serum that will protect against 100 minimal lethal doses of the virus. The tendency of the organism isolated from Hodgkin's disease to clump and resist separation prevented accurate opsonic estimations. Overlooking this difficulty, however, I found but slight increase in opsonins in the immune serum as compared with that from a normal horse. Again, the organism is of such low virulence that it causes no constant acute condition in lower animals which we can inhibit or cure by a fairly definite quantity of serum. Since complement-fixation with bacterial antigens has been successful in the case of gonorrhea, pertussis, and typhoid fever, I eventually employed it in testing the immune serum.

MATERIALS AND TECHNIC OF COMPLEMENT-FIXATION TESTS

Antigens.—The following cultures were used in preparing antigens: 16 strains of diphtheroid bacilli isolated from patients with Hodgkin's disease and lymphosarcoma; 4 strains each of Staphylococcus albus and Staph. aureus; 8 strains of streptococci isolated from tonsils and lymph glands in chronic arthritis; 1 strain of a diphtheroid organism isolated from a lymph gland in chronic arthritis; and 2 strains of B. diphtheriae.

The antigens were prepared as follows:

Antigen 1. The strains of diphtheroid bacilli from Hodgkin's disease were grown on Loeffler's serum agar for 24 hours and then were suspended in sterile normal salt solution. This was centrifugated, the supernatant fluid poured off, the sediment mixed with 15 c.c. sterile salt solution, centrifugated again, the supernatant fluid discarded, and the sediment mixed with 15 c.c. sterile normal salt solution to which 0.5% phenol had been added as a preservative.

Antigen 2. Strains of diphtheroid bacilli grown for from 4 to 8 weeks on serum agar were prepared in the same way..

Antigen 3. A mixture of strains of diphtheroid bacilli grown for 24 hours and for from 2 to 8 weeks was prepared like Antigen 1.

Antigens 4, 5, and 6. These, corresponding to Antigens 1, 2, and 3, were washed and suspended as in the case of Antigen 1, then heated for 1 hour at 60 C.

Antigens 7 and 8. Young and old cultures of 2 strains were prepared as in the case of Antigen 1, as homologous antigens for the patients from whom these strains had been obtained.

Antigen 9. Staphylococcus albus (4 strains) and Staph. aureus (4 strains).

Antigen 10. Streptococcus hemolyticus and S. viridans (8 strains).

Antigen 11. Diphtheroid bacilli (1 strain).

Antigen 12. Bacillus diphtheriae (2 strains).

Antigen 13. Gonococci.

Antigen 14. Syphilitic antigen.

Controls for all antigens, except 13 and 14, were prepared by growing cultures of the respective organisms for from 1 to 4 days on blood-agar slants,

[10] Heinemann and Moore: Jour. Infect. Dis., 1912, 10, p. 294.

washing off the growths with sterile normal salt solution, centrifugating and washing the sediment twice, and then suspending the washed sediment in normal salt solution to which 0.5% phenol had been added as a preservative.

Hemolytic System.—The antichicken hemolytic system was used in all my tests. Washed chicken corpuscles were made up to a 2.5% suspension and used in doses of 1 c.c.; these corpuscles had been sensitized by the addition of twice the hemolytic dose of the hemolysin as found by previous titration. Fresh guinea-pig sera diluted to 8% were used as complement. The complement was titrated each day before the actual tests, and twice the hemolytic dose used with human sera, 3 times it with horse serum. In a recent communication Kolmer[11] has demonstrated that the chicken hemolytic system is more delicate than many others, as no natural amboceptors for chicken corpuscles are found in human sera in the amounts employed in the complement-fixation tests.

All sera were heated to 56 C. for 30 minutes and used in different amounts. Antigen, serum, and complement were incubated for 1 hour at 38 C., the sensitized corpuscles added, the whole again incubated for 1 hour, and then placed in the ice-box over night. Readings were made at the end of the hour and the next morning.

Antigen Titrations.— (1) The anticomplementary dose of each antigen was determined and one-quarter of this used as the antigenic dose. (2) The antigens were titrated with sera of different animals which were supposedly normal and one-quarter of the anticomplementary dose used. By the second method I observed that horse blood and rabbit blood contained more complement-binding substances than did human blood and monkey blood. In order to overcome this increased action of horse and rabbit blood and at the same time have a uniform antigenic dose I increased the quantity of complement to 3 times its hemolytic dose when using horse and rabbit sera. The antigenic doses of both methods then closely paralleled each other. In the sera of some of the rabbits, however, anticomplementary bodies were found in such large quantities that the animals had to be discarded. A 3rd method, that of titration with an immune serum, a method similar to that in use in syphilitic antigenic determinations, was not possible with all antigens in our earlier experiments. By repeated titrations the anticomplementary doses of the antigens were found to remain constant.

The adoption of one-quarter of the anticomplementary dose, in view of the fact that complement was increased to 3 times its hemolytic dose with certain sera in order to overcome all inherent anticomplementary bodies, reduced to a minimum the danger of nonspecific reactions.

In a comparison of the values of various gonococcal antigens Kolmer and Brown[12] assert that their best results were secured with a simple antigen composed of gonococci in sterile normal salt solution plus preservative. In this antigen the endotoxin and the bacterial protein, both of which add to the antigenic properties, are preserved. Since my desire was to obtain a good antigen and not to test various antigens, I adopted that method of preparing antigens which produced best results in gonorrheal complement-fixation. On the other hand, Olitsky[13] in a series of complement-fixation tests with the Corynebacterium hodgkini discarded antigens composed of unfiltered suspensions of the organism as being too anticomplementary and nonantigenic, and used only the filtrates of such suspensions. Since his results differ in no respect from mine, we may assume that both filtered and unfiltered antigens are satisfactory.

[11] Jour. Infect. Dis., 1915, 16, p. 441.
[12] Ibid., 1914, 15, p. 6.
[13] Jour. Am. Med. Assn., 1915, 64, p. 1134.

STANDARDIZATION OF IMMUNE HORSE SERUM

The first serum tested was withdrawn from the horse after the second injection of vaccine. Apparently few antibodies had been developed, as 0.2 c.c. of the serum did not inhibit hemolysis. Titrations were continued at irregular intervals. In September 0.1 c.c. serum caused complement-fixation with 0.04 c.c. antigen. I finally obtained inhibition of hemolysis with 0.0005 c.c. serum plus 0.1 c.c. antigen plus 3 times the hemolytic titer of the complement. Table 1 illustrates the high potency of the serum as determined by the complement-fixation method. The titer has remained at this point for several months.

TABLE 1

Titration of Horse Sera with Diphtheroid Antigen. Antigen — 0.1 c.c. Complement — 3 hemolytic doses

Dose of Serum	Horse 1 Immunized	Horses 50 and 58 Controls	Horse 69 Control	Horse 66 Control
0.0001	75% hemolyzed	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.0003	50% hemolyzed	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.0005	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.0008	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.001	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.05	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.1	Hemolysis inhibited	Hemolysis complete	50% hemolyzed	Hemolysis complete
0.2	Hemolysis inhibited	Hemolysis complete	Hemolysis inhibited	50% hemolyzed

TABLE 2

Titration of Horse Sera with Various Antigens. Antigenic Dose — ¼ Anticomplementary Dose; Complement — 3 Hemolytic Doses; Sera — 0.1 c.c.

	Diphtheroid Bacilli 1	Diphtheroid Bacilli 3	Staphylococci	Streptococci	Diphtheria Bacilli	Gonococci	Syphilitic Antigen
Immunized Horse 1	Hemolysis inhibited	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
Control Horses 50 and 58	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete

In Table 2 the action of control bacterial and lipoidal antigens with immune and control horse sera are shown, demonstrating that the tests

are specific for the organisms used in immunization. Hemolysis resulted in all the control antigens but was inhibited in the diphtheroid antigens when immune horse serum was added. On the other hand, when sera from control horses were mixed with the diphtheroid antigen, hemolysis resulted. Over 25 titrations of the immune horse serum were made with marked inhibition of hemolysis in the higher dilutions which seemed evidence enough that I had produced an antiserum against the diphtheroid organisms isolated in Hodgkin's disease.

This unrefined antiserum was used in the treatment of a few cases of Hodgkin's disease, but the serum reaction was of such a severe nature, even with small doses, that concentration of the serum was immediately suggested. This was accomplished by the technic described. By this method the major portion of the proteins in the horse blood is eliminated and the antibodies are so concentrated that much smaller doses can be administered. I encountered a difficulty, however, in measuring the potency of the refined serum. Concentration had so increased the anticomplementary action that titration by the complement-fixation method was of little value. In amounts of 0.01 c.c. the refined serum was anticomplementary, but not in doses smaller than 0.0005 c.c., the same titer as the unrefined serum. Measured solely by the complement-fixation test, there would apparently be little gain in refining the serum.

Agglutination tests were then undertaken.

Cultures were grown for 24 hours on Loeffler's serum agar, then washed off with sterile salt solution, emulsified, and diluted with sterile salt solution to a satisfactory cloudiness. All tests were made by the macroscopic method. The serum was used in the dilutions shown in Table 3. Five-tenths cubic centimeter serum-dilution plus 0.5 c.c. bacterial emulsion was added to small test tubes, which were then incubated for 2 hours at 37 C., placed in the ice-box for 12 hours, and afterward read. Each set also included a control tube of 0.5 c.c. normal salt solution plus 0.5 c.c. bacterial emulsion.

In 4 tests the control serum caused agglutination at 1 : 640 dilution, the unrefined immune serum at 1 : 2560 dilution, and the refined immune serum at 1 : 5120 dilution. The unrefined immune serum contained approximately 4 times more agglutinin than normal horse serum, and the refined approximately twice as much as the unrefined. While some complement-binding antibodies were apparently destroyed by the process of refining, the agglutinin did not undergo such a marked change.

Table 3 illustrates the intensity of the agglutination reaction in the testing of the sera. While these experiments do not indicate the therapeutic value of the serum, they show that there is immunization produced against this organism with a probable formation of curative antibodies.

More important even than the concentration of the antibodies was the diminution in the nonspecific allergic reactions with the use of the refined serum. Much larger doses of refined serum could be administered to patients than of unrefined, with less of the distressing symptoms of serum disease.

TABLE 3

Agglutination with Horse Sera

Dilution	Unrefined Immune Serum	Refined Immune Serum	Control Serum
1:20	Marked	Marked	Distinct
1:40	Marked	Marked	Slight
1:80	Marked	Marked	Slight
1:160	Marked	Marked	Slight
1:320	Distinct	Distinct	Slight
1:640	Slight	Distinct	Slight
1:1280	Slight	Slight	None
1:2560	Slight	Slight	None
1:5120	None	Slight	None
1:10240	None	None	None

The opsonic value of the serum, measured by the opsonic index, proved to be negative. The index of the immune horse was no greater than that of the control horses. Similar results were obtained by Dr. Garde and Dr. Coleman with sera from patients with Hodgkin's disease, altho in some instances these patients had received numerous doses of vaccine.

EXPERIMENTS ON MONKEYS

In the summer of 1914 I attempted to produce Hodgkin's disease in a rhesus monkey by repeated injections of mixed cultures of the living bacilli which I had at hand, isolated from human cases of the disease. The following report for Monkey 1 gives this experiment in condensed form:

Monkey 1.—Macacus rhesus, male. On July 24, 1914, the animal was injected subcutaneously in the left side of the neck with 1 c.c. of an emulsion containing 8 strains of living bacilli—about 2 billion organisms per cubic centimeter. No lymph glands palpable in axillae or cervical regions. Inguinal lymph nodes slightly enlarged. Leukocytes 12,500. Temperature 101.7.

August 1.—Injected subcutaneously in left side of neck with 1 c.c. of a mixture of 9 strains of living bacilli. A lymph gland approximately 1 cm. in length could be palpated on the upper left side of the neck. Leukocytes 17,800. Erythrocytes 5800. Temperature 102.

August 8.—The same lymph gland still enlarged. Several smaller ones of the cervical chain could be felt. Injected in the same location with 2 c.c. of an emulsion of 13 strains—approximately 5 billion organisms per cubic centimeter. Leukocytes 12,500. Erythrocytes 5,287,000. Temperature 102.4.

August 15.—Injected with 2.5 c.c. of the mixed cultures of 12 strains. Glands of neck small. Leukocytes 14,800. Temperature 102.6.

August 22.—Injected with 2 c.c. of 12 mixed strains—about 7 billion organisms per cubic centimeter—in the cervical region. Glands of neck small. Palpable axillary glands on right side. Leukocytes 11,600. Temperature 102.6.

August 29.—Injected with 3 c.c. of 12 strains—7 billion organisms per cubic centimeter. Lymph glands on right side of neck showed no further enlargement. Monkey appeared well. Leukocytes 13,900. Temperature 102.2.

Injections were made of from 2.5 c.c. to 3 c.c. of 12 strains in the cervical region on Sept. 5; in the left axilla on August 12, Sept. 19, and 26, and Oct. 3; and in the left inguinal region on Oct. 17, with the production of one palpable gland in the left axilla. The enlarged cervical glands disappeared, as did those in the right axilla. The inguinal glands remained about the same. The left axillary glands became much smaller and were just palpable 3 weeks after the last injection. The leukocytic count varied between 12,000 and 17,000. The temperature did not go over 102.4. The monkey reacted very little towards these comparatively large injections. There was no induration around the glands, as described by Bunting and Yates,[4] the individual nodes being freely movable. Monkey 1 died 3 months after the last injection.

The leukocytic count in this monkey was somewhat similar to that of the monkey used by Bunting and Yates.[4] Their monkey from the beginning of the experiment had a higher leukocytic count, averaging 20,450 in 7 counts, while mine averaged 13,500 in 13 counts. As I gave larger and more frequent injections, a higher white count might have been expected but this did not prove to be the case in the actual experiment. Anderson and Neill[14] found the following blood picture as an average of 100 observations on 10 healthy rhesus monkeys of both sexes: Leukocytes 11,192; neutrophiles 46.61%; basophiles (lymphocytes and large mononuclears) 50.65%; eosinophiles, 3.69%; mast cells 0.24%; unclassified, transitionals, 0.8%. In but 1 monkey was the percentage of neutrophiles higher than that of mononuclears; in 1 it was the same; and in the remaining 8 the lymphocytic count was from 6 to 30% higher than that of the neutrophiles. If these counts may be regarded as a norm, there was apparently an increase in the neutrophiles with a corresponding decrease in the lymphocytes in Monkey 1. The percentage of eosinophiles was so irregular that I could not draw any conclusions.

Table 4 illustrates the individual counts and gives the average of Anderson's and Neill's normal counts, together with an average of the 12 counts made by Bunting and Yates. To facilitate counting I have included the large lymphocytes and transitionals under large mononuclears.

Monkey 2.—Injected intravenously with 10 c.c. of the mixed emulsion of 15 strains. It was ill for a few days. Fourteen days later 6 c.c. were given intravenously, after which it was ill for 2 days. Seven days later it was given 5 c.c. intramuscularly in the right thigh and 6 c.c. intravenously in the left femoral vein. It lost its appetite and died on the 5th day. Cultures from the heart blood and from the abscess which developed in the left thigh, contained diphtheroid and colon bacilli. No greatly enlarged glands were found,

[14] Jour. Med. Research, 1915, 33, p. 143.

the largest in the inguinal region of the left side being 7 mm. in size. Histologically, the glands were hyperplastic, as in an ordinary acute infection.

The white blood count in this monkey was high before the experiment, being 24,000, while the temperature was 104. Blood counts were not taken during the experiment.

This monkey died from septicemia due to contamination with B. coli, its death occurring too early for the clinical picture of Hodgkin's disease to develop. However, the 1st monkey showed nothing which clinically resembled Hodgkin's disease.

TABLE 4

BLOOD COUNTS OF MONKEY 1, COMPARED WITH ANDERSON AND NEILL'S NORMAL AVERAGE AND BUNTING AND YATE'S AVERAGE

Date	Leukocytes	Neutrophiles	Small Lymphocytes	Large Mononuclears	Eosinophiles	Mast Cells
7/24	12,500	49.6	43.4	2.7	4.3	.0
8/15	14,800	51.0	42.0	4.8	2.0	.2
8/29	13,900	59.2	35.0	4.6	1.0	.2
9/ 5	12,000	59.0	32.5	7.1	1.1	.3
9/12	12,500	68.8	22.5	5.1	3.6	.0
9/19	12,000	53.4	40.6	3.1	2.6	.3
9/26	17,000	59.0	31.3	6.3	3.3	.1
10/ 3	13,500	55.5	38.5	5.5	0.5	.0
10/ 7	12,600	52.2	39.0	7.8	1.4	.4
10/24	13,200	61.3	29.4	3.0	6.7	.2
Average..............	13,500	56.9	35.47	4.79	2.75	.17
Anderson and Neill's average...........	11,192	41.61	54.45		3.69	.24
Bunting and Yate's average...........	20,450	46.0	40.9	10.73	2.6	.35

The technic previously described for the standardization of the horse-serum complement-fixation tests, employed for the monkeys gave somewhat parallel results. After the series of injections the serum of Monkey 1 fixed complement when 0.05 c.c. of serum was used. Before the experiment hemolysis had occurred with 0.2 c.c. of serum. In the case of Monkey 2 serum from blood drawn at the time of the last injection prevented hemolysis in doses of 0.005 c.c. Before the experiment 0.3 c.c. had failed to prevent hemolysis. Agglutination and opsonic tests were not tried with the sera of the monkeys.

EXPERIMENTS ON RABBITS

Before the inoculation of rabbits was commenced, complement-fixation tests were made on 5 animals, the sera of 2 of which fixed complement in doses as small as 0.05 c.c., of 1 in dose of 0.1 c.c., while the sera of the remaining 2 gave complete hemolysis in doses of 0.2 c.c.

The latter animals were used for immunization. The phenomenon of complement-binding when normal rabbit serum is added to bacterial antigens is well known. Olitsky[13] in testing a series of rabbits before immunization with Hodgkin's bacilli obtained the same result and discarded animals which bound complement in high dilutions in the presence of antigen.

Immune rabbit sera were prepared as follows: 4 billion organisms of a mixed emulsion of live bacilli were injected subcutaneously; followed in 5 days by 10 billions, then by 5 doses increasing from 4 to 10 billions, intravenously every 5 days. Serum was withdrawn at the end of 10 days and found to bind complement completely in doses of 0.0003 c.c. The organism appeared to be entirely avirulent for rabbits, but produced a high degree of immunity as measured by complement-fixation.

TOXIN-PRODUCTION

Two Erlenmeyer flasks of glucose broth were each inoculated with 12 strains of diphtheroid bacilli isolated from Hodgkin's disease. One was incubated for 5 days, the other for 10 days. At the end of these periods both were centrifugated for 20 minutes at 3400 revolutions a minute, and then 5 c.c. of the supernatant broth injected into the peritoneal cavities of two 250-gram guinea-pigs. As neither of the guinea-pigs appeared in the least discomfited by the large amounts of broth, we may judge that soluble toxin in appreciable amounts was not produced. These findings agree with the results obtained by others[15, 16, 17] as to toxin-production by diphtheroid organisms.

COMPLEMENT-FIXATION IN PATIENTS WITH HODGKIN'S DISEASE

In this series of studies I was fortunate enough to have the sera from 10 cases (9 of which were in Dr. Billings' service) diagnosed as Hodgkin's disease. In 9 of these, histologic examination of lymph glands confirmed the clinical diagnosis and cultures from the majority of the cases resulted in the growth of a pleomorphic diphtheroid organism.

The technic in these tests was the same as that described. From 0.1 to 0.6 c.c. of the patient's serum was used, the usual amount being from 0.1 to 0.2 c.c. Sera were obtained from the following cases of Hodgkin's disease. (The histories are not complete, giving only the

[15] Hamilton: Jour. Infect. Dis., 1904, 1, p. 690.
[16] Clark: Jour. Infect. Dis., 1910, 7, p. 335.
[17] Fox: Jour. Med. Research, 1915, 32, p. 325.

data which are related to the tests.   All treatments other than with
vaccine and serum are omitted.)

Case 1.—Clinical diagnosis, Hodgkin's disease. Cervical glands first enlarged
2 years before first test. Nine months after appearance of symptoms a gland
had been removed and diagnosed as Hodgkin's disease by the Mayo Clinic.
Diphtheroid bacillus had been isolated from a gland removed at the Presbyterian
Hospital, Chicago, and this made into an autogenous vaccine. Histologic
examination of the gland had confirmed the previous diagnosis. Patient had
had 14 doses of vaccine before the first complement-fixation test. The 6 tests
covered a period of 5 months. Previous to the last test the patient had received
over 50 doses of nonsensitized and sensitized autogenous vaccine, ranging from
10 million to 1 billion organisms each. With this were given several doses of
immune horse serum. The patient left the hospital much improved, having
gained 19 pounds in weight.

Case 2.—Clinical diagnosis, Hodgkin's disease. Pathologic diagnosis of
excised lymph gland, Hodgkin's disease. The organism isolated from the
gland was a coccus form. Duration of disease about 1 year. Seven doses
of a mixed Hodgkin's and staphylococcus vaccine had been administered before
the first test, 15 before the last, together with several doses of immune horse
serum. Some improvement noted. The tests covered a period of about 2
months.

Case 3.—Clinical diagnosis, Hodgkin's disease. The symptoms had been
present over 2 years. A pleomorphic diphtheroid organism had been isolated
from an excised lymph gland and at the same time a histologic diagnosis of
Hodgkin's disease had been made. Over 12 vaccinations had been made before
the first test, and 9 before the last test. Three tests were made in a period
of 6 weeks. The patient died a week after the last test.

Case 4.—Clinical diagnosis, Hodgkin's disease. The first symptom had
appeared over 2 years before. Cultures from excised lymph glands had been
sterile. Histologic diagnosis, Hodgkin's disease. No vaccine had been given
before the first test, but over 40 doses of mixed diphtheroid bacilli ranging from
1 to 5 million organisms each, in addition to immune horse sera, had been
injected before the last test. The 5 tests covered a period of 3 months.

Case 5.—Clinical diagnosis, Hodgkin's disease. The symptoms had lasted
18 months before the first serum was obtained and about 20 vaccinations had
been made. The histologic diagnosis had been Hodgkin's disease and a diph-
theroid organism had been isolated from the lymph glands. Over 50 injections of
nonsensitized and sensitized autogenous vaccine with immune horse serum
had been administered previous to the last of 6 tests. The serum was exam-
ined once a month for 5 months. In this case I used not only the mixed anti-
gens but an antigen prepared from the bacillus isolated from the patient's
diseased lymph gland. The patient received 1 dose of vaccine intravenously.
Agglutination tests were also made on this serum.

Case 6.—Clinical diagnosis, Hodgkin's disease. The first symptoms had been
noted 6 months before the test. A diphtheroid organism had been twice isolated
from the lymph glands. One dose of vaccine had been injected before the test.
The patient died.

Case 7.—Clinical and histologic diagnosis, Hodgkin's disease. The symp-
toms had persisted for 3 years. No vaccine therapy had been employed previous
to the first serum examination. Nine doses of autogenous vaccine were given
before the last test.

Case 8.—Clinical and histologic diagnosis, Hodgkin's disease. A diphtheroid bacillus had been isolated from an excised lymph gland. No vaccination had been made previous to the first test. An antigen prepared from the organism isolated from the patient was also used. Tests for agglutinin were also made on this serum.

Case 9.—Clinical and pathologic diagnosis, Hodgkin's disease and congenital lues. The glands had been enlarged for 18 months. The test was made before any vaccine had been given. A positive Wassermann was repeatedly obtained with this serum.

Case 10.—Clinical diagnosis, Hodgkin's disease. The symptoms had been obvious for over 18 months. No vaccine had been given before the one test. The glands have not yet been examined histologically.

The sera from several cases diagnosed clinically as Hodgkin's disease, but which microscopic examination of glands proved to be lymphosarcoma or tuberculosis, were tested. One of these, a lymphosarcoma, requires special consideration, as a pleomorphic diphtheroid bacillus morphologically and culturally identical with those obtained from the Hodgkin's glands was isolated from the glands.

Case 11.—Clinical diagnosis, Hodgkin's disease. Histologic diagnosis, lymphosarcoma of cervical glands. The condition had been progressing for 11 months. The patient had had several doses of autogenous vaccine before the first complement-fixation test and over 40 before the last. Immune horse serum was also employed. The tests extended over 2 months. The patient has since died. This case may be classed immunologically with the cases of Hodgkin's disease.

The complement-fixation tests were made at irregular intervals over a period of more than 5 months in 2 cases; 3 months in 1 case; 2 months in 1 case; 1 month in 3 cases; and examination was made but once in 3 cases—in all, a total of 31 different examinations. In no instance did I find inhibition of hemolysis with any of the various diphtheroid antigens or with any of the control antigens except in the case of the Wassermann reactions (Case 10). Olitsky[13] obtained similar negative results in a series of 6 cases.

In Cases 5 and 8 an antigen was prepared from the organisms isolated from the patients and the patients' sera tested against these homologous antigens. The reactions were negative in both cases.

Apparently no complement-binding antibodies had been produced by the vaccinations. Three individuals were tested before they had received any vaccine, and 1 after but 1 dose. Others had had from 7 to 10 doses before the first test and some over 50 before the last test. One dose of vaccine contained from 10 million to 1 billion organisms. One patient had a dose of vaccine intravenously which produced a

severe general reaction, but tests failed to show any positive comple-
ment-fixations.

In Case 10 a positive Wassermann reaction was obtained several
times in different laboratories. This case was diagnosed clinically as
congenital lues plus Hodgkin's disease. The histologic diagnosis was
Hodgkin's disease. As in all other cases there was complete hemolysis
with the lipoidal antigens, we may assume that Hodgkin's disease and
lues are not closely related. It is of interest that negative Wassermanns
were obtained for the 8 cases of myelogenic and lymphatic leukemia,
and for 2 cases of lymphosarcoma, as well as for all other serum con-
trols except the syphilitics.

TABLE 5

COMPLEMENT-FIXATION TESTS IN HODGKIN'S DISEASE

No. of Cases	Diagnosis	No. of Tests	Result	Remarks
10	Hodgkin's disease......	31	Hemolysis	Six cases had vaccine
2	Lymphosarcoma.......	6	Hemolysis	Diphtheroid bacillus isolated from 1 case
6	Myelogenic leukemia....	12	Hemolysis	
2	Lymphatic leukemia....	2	Hemolysis	
4	Tuberculosis...........	5	Hemolysis	Three cases of tuberculous lymphadenitis
2	Asthma...............	3	Hemolysis	
6	Syphilis..............	6	Hemolysis	
1	Chronic arthritis.......	1	Hemolysis	
1	Chronic arthritis.......	1	Hemolysis	
4	Normal................	6	Hemolysis	
1	Immunized horse.......	25	Hemolysis inhibited	Injected with live bacilli for 6 months
5	Control horses..........	26	Hemolysis	Three diphtheria antitoxin horses were included

Since staphylococci had been isolated from the diseased glands in
Hodgkin's disease,[6] it was thought possible that some of these sera
might react positively with staphylococcus antigen. This was not found
to be true.

As controls, sera from the following diseases (see Table 4) were
tested with the several antigens: myelogenic leukemia, 6 cases;
lymphatic leukemia, 2 cases; lymphosarcoma, 2 cases; tuberculous
lymphadenitis, 3 cases; pulmonary tuberculosis, 1 case; asthma, 2 cases;
chronic arthritis, 1 case; syphilis at various stages, 6 cases; chronic
nephritis, 1 case; and normals, 4 cases. The cases of leukemia were
selected because of the close relationship of that disease to Hodgkin's
disease or pseudoleukemia, and in view of the isolation by Steele[18] and
Simon and Judd[19] of diphtheroid organisms from lymphatic leukemia;

[18] Boston Med. and Surg. Jour., 1914, 170, p. 123.
[19] Jour. Am. Med. Assn., 1915, 64, pp. 20, 1630.

the cases of lymphosarcoma were selected for similar reasons; tuberculosis, because of the clinical resemblance of tuberculous lymphadenitis to Hodgkin's disease, and because similar organisms had been isolated in this disease; chronic arthritis, because diphtheroid organisms had been found in lymph glands in this condition; syphilis was selected for the purpose of ascertaining whether a positive syphilitic blood would react positively with the bacterial antigens used. The tests with homologous antigens were negative in every case.

AGGLUTINATION TESTS ON PATIENTS WITH HODGKIN'S DISEASE

The sera of Cases 5 and 8 were examined for agglutinin. In both cases bacterial emulsions consisting of organisms isolated from the patients as well as mixed emulsions of bacilli from other cases of Hodgkin's disease were tested. No higher agglutination could be observed in the patient's sera than in the normal controls, either with the homologous, or with the mixed emulsions. In a personal communication from Dr. Garde and Dr. Coleman I learn that they have made several agglutination tests on other pseudoleukemic patients using homologous antigens with negative results.

SUMMARY

Horses can be immunized by repeated intravenous injections of pleomorphic diphtheroid bacilli isolated from the lymph glands in Hodgkin's disease.

In complement-fixation tests this immune serum bound complement when used with these same organisms as antigens in amounts as small as 0.0005 c.c. Complete hemolysis occurred with control antigens of staphylococci, streptococci, diphtheria bacilli, gonococci, and lipoidal extracts and 0.1 c.c. of the immune serum. Complete hemolysis resulted when control horse sera were added to the diphtheroid antigen in amounts of 0.1 c.c.

Agglutinin was found to be increased fourfold by the immunization.

By refining this serum according to the methods employed in concentrating diphtheria antitoxin, it was found that altho no definite increase in the complement-fixing antibodies could be demonstrated, an increase in agglutinin could be shown. The refined serum produced fewer allergic reactions than the whole serum.

Monkeys were immunized with the same organisms by both subcutaneous and intravenous inoculations. An increase in complement-

binding amboceptors was found in the serum after the injections.   By similar methods immune sera were obtained from rabbits.

Soluble toxins were not formed by these organisms when grown in glucose broth.

Complement-fixation tests were made on 10 individuals having Hodgkin's disease and in no case was there inhibition of hemolysis with antigens of the mixed cultures isolated from Hodgkin's disease. In 2 cases antigens prepared from organisms cultured from the patients' glands were used, with negative results.

Vaccination with these organisms did not appear to increase the complement-binding antibodies in patients.

Complement-fixation tests on the sera of these patients with control antigens were negative in all except one instance in which positive Wassermanns were repeatedly obtained.

Agglutination experiments with the sera of 2 patients were likewise negative.

Complement-fixation tests made on sera from cases of lymphosarcoma, lymphatic leukemia, chronic arthritis, and tuberculosis, diseases in which diphtheroid organisms have been isolated, all reacted negatively with the various antigens used.

A STUDY OF GAS-PRODUCTION BY DIFFERENT STRAINS OF BACILLUS ABORTIVO-EQUINUS *

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In our work on the etiology of infectious abortion in mares,[1] we found that the organism causing this disease, Bacillus abortivo-equinus, varied in its physiologic property of splitting some of the sugars, especially lactose and saccharose.

De Jong[2] makes the positive statement that the bacillus, which he isolated from the uterine exudate of aborting mares, does not ferment lactose, but that it does ferment saccharose. Meyer and Boerner[3] also state that the organism causing infectious abortion in mares does not ferment lactose and saccharose. Later, Van Heelsbergen,[4] working with De Jong, notes that the organism does not ferment lactose and saccharose.

Therefore, in order that more definite conclusions might be arrived at, considerable work needed to be done on this variable fermenting propensity, and the problem was taken up by us in the hope that some positive statement could be made concerning the gas-production of this organism.

As has been stated,[5] our laboratory placed this organism in Subgroup II of the colon-typhoid group, to which Bacillus enteritidis and the paracolon bacilli belong. As these organisms are all classified in the same subgroup, the reason for employing them in a comparative study of this nature can be readily understood.

It is interesting to note what some of our recognized authorities write concerning the splitting of lactose by bacilli classified in this subgroup. Take, for example, B. enteritidis (Gaertner). According to Buchanan,[6] "lactose is not fermented by the typical strains, although some strains have been reported by a few investigators to ferment this

* Received for publication November 24, 1915.

[1] Good and Corbett: Jour. Infect. Dis., 1913, 13, p. 53.
[2] Centralbl. f. Bakteriol., I, O., 1913, 67, p. 148.
[3] Jour. Med. Research, 1913-14, 29, p. 334.
[4] Inaugural Dissertation, 1913.
[5] Good: Bull. Ky. Agr. Exper. Sta., No. 165, 1912, p. 280.
[6] Veterinary Bacteriology, 1911, p. 269.

sugar, and the statement is commonly current in texts." Chester,[7] on the other hand, makes the unqualified statement that B. enteritidis forms gas in lactose broth. Herzog[8] also asserts that it ferments lactose, while Jordan[9] states that no gas or acid is formed from lactose.

J. Henderson Smith[10] has shown that organisms without the power to ferment certain carbohydrates may be so changed, after being grown for a number of generations on, or in, media containing these sugars, that they become true fermenters. He says: "In the year 1906, M. Neisser recorded the occurrence of a coli-form organism, which, while primarily a non-fermenter of lactose, could give rise to a fermenting strain when grown on lactose-agar. This organism was very carefully and thoroughly studied by Massini, who proved that the fermenting strain was not a contamination but was really derived from the original non-fermenting organism, and arose upon lactose-agar as a variant with a new character which bred true. Since then, numerous instances have been recorded by Burk,[11] Jacobsen,[12] Twort,[13] Müller,[14] and others, of members of the Colon-Typhoid group which displayed a similar capacity of varying towards one or more of the carbohydrates, and the fact of bacterial variation in this direction is now beyond dispute."

As the nutrient agar on which we grow our cultures is made from fresh beef-meat infusion containing the natural muscle sugars, and not from beef extract, it was thought that perhaps Bacillus abortivo-equinus quickly accommodated itself to an environment of lactose and acquired the ability to ferment this sugar. We have proved this not to be the case, not only by using cultures of the first generation, but by making inoculations of the lactose broth directly from the tissues of the original material. Dilutions of this same material in nutrient agar gave a pure culture of the organism. After the growth had fermented the lactose broth, dilutions from this broth in agar yielded, as far as we could determine, a pure culture, thus proving that the organism in some cases possesses as an original physiologic characteristic the ability to ferment lactose, and that the resultant gas-production is not caused by a contaminator.

[7] A Manual of Determinative Bacteriology, 1901, p. 207.
[8] A Text-book on Disease Producing Micro-organisms, etc., 1910, p. 452.
[9] A Text-book of General Bacteriology, 1914, p. 262.
[10] Centralbl. f. Bakteriol., I, O., 1913, 68, p. 151.
[11] Arch. f. Hyg., 1908, 65, p. 235.
[12] Centralbl. f. Bakteriol., I, O., 1910, 56, p. 206.
[13] Proc. Roy. Soc., 1907, 79, p. 329.
[14] Centralbl. f. Bakteriol., R., 1908, 42 p. 57.

The Smith fermentation tubes[15] were used in the first fermentations carried on by us. Since with these tubes it is possible to measure with precision the amount of gas formed in the upright arm, they are valuable in the quantitative determination of gas-production. The Smith tubes, however, require a large amount of media, and they are fragile, and cumbersome.

The inverted vial is a simple combination, merely a small inverted tube, sealed at one end, in a test tube. The handling of this device, as can readily be seen, is the same as that of a test tube, and it is therefore especially convenient for extended work with gas-production. It is not possible, however, to measure with much exactitude the amount of gas formed. However, in our experiments the vials were all made of the same caliber glass tubing and were all as near the same length as was possible with ordinary laboratory technic. The inverted tubes were therefore practically identical and exact comparisons could be made as to the amount of gas formed.

W. W. Brown[16] concludes from a comparative study of the Smith fermentation tube and the inverted vial in the determination of sugar-fermentation that inverted vials are more efficacious in the low dilutions than the fermentation tubes, and that fermentation tubes are more efficacious in higher dilutions than the inverted vials. Brown's conclusions are based on his work with oysters grown in beds polluted by city sewage. He inoculated his sugar media with varying amounts of shell liquor obtained from the oysters. In all of our work, the sugar media were heavily inoculated, and therefore, if Brown's conclusions hold true, with our technic the inverted vial was the more efficacious.

In order that we might satisfy ourselves as to the efficacy of the inverted vial in registering small amounts of gas, the two systems were given a thorough comparative test. Test tubes containing inverted vials and the regulation Smith fermentation tubes were filled with lactose broth of the same lot. The tubes, both the Smith tubes and the inverted vials, were inoculated with a strain of B. abortivo-equinus taken from the same subculture. A number of controls were also used; that is, several tubes of the media were not inoculated, but were incubated and otherwise treated in the same manner as those in which the organism had been placed. The tubes were incubated at 37.5 C. Observations made at the end of 24, 48, and 72 hours showed that

[15] Smith: The Fermentation Tube, 1893.
[16] Am. Jour. Pub. Health, 1913, 3, p. 701.

30% more of the inverted vials registered gas than of the Smith tubes, while all the control tubes of both kinds, remained negative.

By careful measurement we find that the average amount of gas produced in lactose broth by B. abortivo-equinus in Smith fermentation tubes, is about 2%. The amount of gas formed from lactose and saccharose sugars by B. abortivo-equinus was always very small. It was thought that possibly the small bubble arising after inoculation of the tubes with this organism was caused by a physiologic change in the medium, due either to the mechanical disturbance produced by whipping the broth with the platinum needle at the time of the introduction of the culture. or to expansion and later contraction of the contents of the inverted vial, caused by the difference in temperature between incubator and laboratory. The temperature of the incubator is always 37.5 C., while that of the laboratory varies between 20 and 25 C.

A long series of tests was made to determine whether the bubble was of physical or chemical origin. A number of tubes were inoculated with different strains of B. abortivo-equinus. A much larger number were whipped with a sterile platinum needle, the treatment being the same as that accorded the inoculated tubes, save the sterility of the inoculating needle. These tubes, together with a great many control tubes, were placed in the incubator. The control tubes were inverted vials filled with media identical with that in the inoculated tubes. The cotton plugs in these tubes were not removed from the time of sterilization of the tube and its contents until the test was over. The control tubes in this case were used as a check on the original media. The test was observed every day for several days, and usually most of the inoculated tubes showed the characteristic bubble, but in no instance was even a minute bubble observed in the tubes containing the media whipped with the sterile needle, or in the control tubes.

From this test we have concluded that the small bubble, which we are wont to term "the characteristic bubble," is without doubt the result of fermentation; altho very small, usually about 2% of the media being displaced, this bubble is the result of chemical change. Therefore we believe that B. abortivo-equinus does ferment lactose in a majority of trials, and saccharose, in some cases, to a slight degree, and that the characteristic bubble encountered in our tests is not of physical, but of chemical, origin.

Cultural media prepared by commercial companies are not used in our laboratory. The media for the tests are prepared by us according

to Jordan.[17]   Five hundred grams of minced lean beef are placed in
1000 c.c. of distilled water and kept in the ice-box over night.   The
liquor is then strained—the juice having been well pressed out of the
meat—and boiled for half an hour to coagulate the albumins.   These
are then filtered out.   Meat usually contains a slight amount of muscle
sugar.   A simple method of removing the muscle sugar is that devised
by Theobald Smith: From 10 to 20 c.c. of a pure young broth culture
of B. coli are added to the infusion of meat and the whole incubated
18 hours at 37.5 C.   The broth is then placed in the autoclave and sub-
jected to from 20 to 25 pounds pressure to kill the organism.   A series
of fermentation tubes is filled with this supposedly sugar-free broth,
inoculated with an active gas-producing B. coli, and incubated at 37.5
C. for 24 hours.   If no gas is formed, we are reasonably certain that
all the fermentable carbohydrates have been removed.   The fluid is
now made up to 1000 c.c. with distilled water, and 10 gm. of Witte's
peptone are carefully stirred in and dissolved by heating.   The broth,
which at this stage is generally markedly acid, is then titrated and
adjusted while hot to a neutral reaction by the addition of the required
amount of a normal solution of sodium hydroxid.   The broth is then
heated in the autoclave to 120 C., allowed to cool again to bring down
the precipitate caused by change of reaction, and filtered.   The special
broth media are now finally prepared by the addition of 1% of the
desired sugar or other carbohydrate to the sugar-free broth.   Five
cubic centimeters of the special broth medium are pipetted into a tube
containing an inverted vial and sterilized in the Arnold steam sterilizer
by the discontinuous method for 3 successive days.   The following
sugars have been used in our fermentation tests: xylose, adonite, rham-
nose, raffinose, arabinose, sorbose, sorbite, dulcite, dextrose, mannite,
maltose, saccharose, and lactose.   As the ability of B. abortivo-equinus
to split the last two sugars named was questioned, the greater part of
the work was done with saccharose and lactose.

All strains of B. abortivo-equinus used in these tests were isolated
by this laboratory from cases of infectious abortion in mares in several
different studs located in the Bluegrass Region.   The strain of B.
enteritidis (Gaertner) was secured from Dr. Biehn, of Chicago.   The
strain of the paracolon bacillus was presented to us by Dr. Surface,
who personally brought the culture from the laboratory of Prof. Dr.
C. O. Jensen, of Copenhagen.   For convenience, the organisms will be

[17] A Text-book of General Bacteriology, 1912, p. 29.

referred to by number. Numbers 1 to 16 were Bacillus abortivo-equinus, isolated as follows:

1. From the aborted male fetus of a mare.
2. From the uterine exudate of an aborting mare.
3. From the aborted female fetus of a jennet.
4. From the aborted fetus of a mare.
5. From the fetal membranes of an aborting mare.
6. From the fetal membranes of an aborting mare.
7. From the aborted male fetus of a mare.
8. From the aborted male fetus of a mare inoculated experimentally with the organism.
9. From the aborted male fetus of a mare.
10. From the aborted male fetus of a mare.
11. From the uterine exudate of an aborting mare.
12. From the uterine exudate of an aborting mare.
13. From the aborted male fetus of a mare.
14. From the genital discharge of a mare giving birth to a very weak living foal, in a stud where infectious abortion existed.
15. From the uterine exudate of an aborting mare.
16. From the uterine exudate of an aborting mare.
17. A mixed culture of the Bacillus abortivo-equinus consisting of Strains 2, 3, 5, 6, 7, 11, 12, 13, and 15.
345. Bacillus enteritidis (Gaertner) secured from Dr. Biehn.
597. A paracolon bacillus isolated from a case of diarrhea in a calf. This bacillus had been secured from Dr. Surface.

By reason of careful laboratory practice we are comparatively certain that our observed results were caused by the inoculations and not by contamination. All glassware after being washed with soap and water is rinsed and dried and sterilized with dry air at 200 C. After being filled with the media the tubes are again sterilized by the discontinuous method in the Arnold steam sterilizer. Culture tubes are never opened, except in a dust-proof compartment especially constructed for this purpose, the walls of which are kept saturated with glycerin. In almost all instances the inoculations were made from 18-hour-old plain-agar cultures, the material being taken from the drop of condensation. Check tubes were used in every series of fermentation tests.

The following tables give the results of our fermentation tests. In Table 1 are presented observations made when 1% lactose broth was inoculated severally with the 16 different cultures of B. abortivo-equinus, and one (No. 17) mixed culture of Strains 2, 3, 5, 6, 7, 11, 12, 13, and 15. Table 2 gives observations made when 1% saccharose was used instead of 1% lactose. All observations were made after 60 hours' incubation at 37.5 C.

TABLE 1

GAS-PRODUCTION IN 1% LACTOSE BROTH BY DIFFERENT STRAINS OF BACILLUS ABORTIVO-EQUINUS

Strain	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas	Average Percentage of Gas When Produced	Average Pecentage of Acid
1	14	8	6	1.5	.3
2	6	6	..	3.0	.3
3	12	6	6	1.5	.2
4	10	5	5	1.5	.2
5	6	6	..	3.0	.2
6	7	7	..	2.5	.4
7	5	5	..	3.0	.4
8	8	8	.	3.0	.5
9	5	5	..	3.0	.5
10	8	4	4	1.5	.2
11	4	4	..	2.0	.2
12	3	3	..	2.0	.3
13	5	3	2	2.0	.2
14	8	3	..	2.0	.2
15	4	4	..	2.0	.2
16	4	4	..	2.5	.2
17	12	12	..	5.0	.3
Total.......	116	93	23	...	...

TABLE 2

GAS-PRODUCTION IN 1% SACCHAROSE BROTH BY DIFFERENT STRAINS OF BACILLUS ABORTIVO-EQUINUS

Strain	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas	Average Percentage of Gas When Produced	Average Pecentage of Acid
1	11	2	9	2.0	.2
2	4	2	2	1.8	.15
3	7	2	5	1.5	.15
4	4	1	3	2.0	.2
5	2	1	1	1.0	.2
6	2	1	1	1.2	.25
7	2	2	..	2.0	.2
8	3	1	2	1.5	.2
9	2	2	..	2.0	.25
10	5	2	3	1.8	.2
11	2	2	..	2.2	.25
12	2	1	1	2.5	.25
13	2	2	..	2.0	.2
14	2	1	1	1.5	.2
15	2	2	..	2.0	.2
16	2	2	..	2.0	.15
17	2	2	..	1.8	.2
Total.......	56	28	28	...	...

TABLE 3

Comparison of Bacillus Abortivo-equinus, Bacillus Enteritidis (Gaertner), and the Paracolon Bacillus in Regard to Gas-Production in 1% Lactose Broth

Organism	Source	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas
B. abortivo-equinus......	Aborting mares.......	116	93	23
B. enteritidis..............	Not known............	9	6	3
Paracolon bacillus........	Calf diarrhea.........	2	..	2

TABLE 4

Comparison of Bacillus Abortivo-equinus, Bacillus Enteritidis (Gaertner), and the Paracolon Bacillus in Regard to Gas-Production in 1% Saccharose Broth

Organism	Source	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas
B. abortivo-equinus......	Aborting mares.......	56	28	28
B. enteritidis..............	Not known............	7	1	6
Paracolon bacillus........	Calf diarrhea.........	5	..	5

TABLE 5

Gas-Production on Other Sugars

Sugar	B. Abortivo-equinus 1	B. Abortivo-equinus 3	B. Abortivo-equinus 4	B. Abortivo-equinus 10	B. Enteritidis (Gaertner) 345	Para-colon Bacillus 597
Xylose.....................	+	+	+	+	+	+
Adonite...................	—	—	—	—	—	—
Rhamnose.................	—	—	—	—	—	—
Raffinose.................	+	+	+	+	+*	+*
Arabinose.................	+	+	+	+	+†	—
Sorbose...................	—	—	—	—	—	—
Sorbite...................	+	+	+	+	+	+
Dulcite...................	+	+	+	+	+	+
Glucose...................	+	+	+	+	÷	÷
Mannite............	+	+	+	+	+	—
Maltose...................	+	+	+	+	..	..

The sign + indicates the production of gas; the sign —, no gas.
* Small amount of gas was noted after 3 days' incubation.
† An extremely small bubble of gas in one instance and no gas in another test.

From the tables it will be seen that of 116 tests with B. abortivo-equinus in lactose, 93 show an average of 2% gas-production and 23 no gas. With the same organism in saccharose, 28 of the tests are positive, showing a little less than 2% gas, while 28 are negative for gas but show a slight amount of acid, an average of about 0.3%.

The question now arises as to what the cause of this variance is. It would seem that if gas is produced in some instances, it would be in all cases, if the same organism is used and the same cultural medium

and technic are employed. This is not always the case, and if variants of this organism are not the cause, we are at the present time unable to suggest an explanation of the phenomenon. When our tests are made in duplicate and triplicate, the cultural medium is of the same lot, the inoculating material comes from the same culture, and the tubes are treated exactly alike; yet after incubation we often find that the duplicates differ, one being positive and the other negative. If any gas is produced, be it ever so slight, the test must be recognized as positive. When the test is made in triplicate, one of the tubes often fails to agree with the other two.

The culture of B. enteritidis (Gaertner) produced approximately 2% gas in lactose in 75% of the trials; in saccharose a slightly smaller amount of gas was produced in 1 of 7 trials. The strains of paracolon bacilli used did not produce gas in either lactose or saccharose.

In these tests the four strains of B. abortivo-equinus produced the following average percentages of gas in the carbohydrates which were fermented: xylose 51%; raffinose 39%; arabinose 59%; sorbite 82%; dulcite 95%; glucose 74%; mannite 81%. With these same materials, B. enteritidis (Gaertner) produced the following amounts of gas: xylose 30%; arabinose, a minute bubble in one instance and none in another; raffinose 8%; sorbite 85%; dulcite 5%; glucose 60%; and mannite 80%. The paracolon bacilli used produced 5% gas in xylose, 7% in raffinose, 85% in sorbite, 75% in dulcite, 60% in glucose, 80% in mannite and no gas in arabinose. In these tests no gas was produced by the four strains of B. abortivo-equinus used, or by B. enteritidis (Gaertner) and B. paracolon, in adonite, rhamnose, and sorbose.

From these tests, it would seem that arabinose, raffinose, and dulcite could be used to good advantage in differentiating B. abortivo-equinus from B. enteritidis (Gaertner) and the types of paracolon bacilli used, as in these tests only a most minute bubble of gas was formed in arabinose by B. enteritidis and none in another, and no gas was formed by the paracolon bacillus. An average of 40% gas was produced by B. abortivo-equinus in raffinose, while only 8% gas was produced in this medium by the other organisms. However, these results are not in accord with the general literature on the subject, in that raffinose is not fermented by organisms belonging to intermediate groups of the colon-typhoid group.[18] The fermentation in dulcite by B. abortivo-equinus is but little during the first 24 hours, after which it

[18] Besson: Practical Bacteriology, Microbiology and Serum Therapy, p. 434.

proceeds rapidly and in some cases to such an extent as to empty the medium in the inverted vial. Meyer[19] also states that the reaction on dulcite can be used for differentiation.

CONCLUSIONS

The inverted vial was as efficacious in our work as the Smith fermentation tube.

The bubble, or the small amount of gas, encountered so often in our fermentation tests with Bacillus abortivo-equinus in lactose and saccharose broth is not of physical, but of chemical, origin.

Bacillus abortivo-equinus produced approximately 2% gas in lactose in 80% of 116 trials, and in saccharose slightly less than 2% gas in 50% of 56 trials.

The average gas-production by the strain of Bacillus enteritidis (Gaertner) was about 2% in lactose in 80% of the trials, and a slightly smaller amount in saccharose in 1 of 7 trials. The strain of the paracolon bacillus used did not ferment lactose or saccharose, a fact which is in accord with the literature on the subject.

Bacillus abortivo-equinus may or may not produce gas in 1% lactose or saccharose broth, even varying in this respect in duplicate and triplicate tests.

Bacillus abortivo-equinus possesses as an original physiologic characteristic the ability, in most cases, to ferment lactose to a small extent, and also, in some cases, to ferment saccharose to a less extent. This characteristic in all probability has not as yet been accentuated by environment.

Lactose and saccharose broth can be employed to good advantage in laboratory routine for differentiating Bacillus abortivo-equinus from the colon bacillus, as the gas, when produced, is small in amount; and, in all probability, dulcite and perhaps raffinose can be used to advantage in differentiating Bacillus abortivo-equinus from other members of Subgroup II of the colon-typhoid group, but absolute proof as to its identity can only be secured through the use of other tests, such as those for further cultural characteristics and the complement-fixation and agglutination tests.

[19] Jour. Med. Research, 1913-14, 29, p. 325.

A NEW CULTURE MEDIUM FOR THE ISOLATION OF BACILLUS TYPHOSUS FROM STOOLS *

PLATE 20

J. E. Holt-Harris and Oscar Teague

From the Quarantine Laboratory, Health Officer's Department, Port of New York

Of the numerous methods which have been recommended for the isolation of typhoid bacilli from feces the one at present most widely used is that devised by Endo in 1903. It allows the development of a high percentage of the typhoid organisms inoculated on it, but does not inhibit to any practical extent the growth of those fecal bacteria which develop on ordinary nutrient agar. The great value of the method lies in its sharp differentiation between the lactose-fermenting organisms and those organisms which do not ferment lactose. The colonies of the lactose-fermenting organisms are colored red after 24 hours' incubation, while the other colonies remain colorless. We have found that better results are obtained if both lactose and saccharose are added to the Endo medium, since certain members of the colon-bacillus group ferment saccharose more rapidly than lactose and hence their colonies take on the red color sooner and can no longer be regarded as slow colonies; the typhoid colony is without color in 24 hours, as on the Endo medium containing lactose alone.

The chief disadvantage of the Endo medium lies in the fact that the red color is not confined to colonies of B. coli themselves, but spreads out through the medium adjacent to them. If the colonies of B. coli are close together the whole plate soon becomes red and then colorless colonies on it can no longer be distinguished from the red ones. We have tested a great number of stains separately and in combinations of varying strengths with the view of overcoming this difficulty while at the same time preserving the effectiveness of the medium for the sure growth of typhoid. In every instance where the acid or the basic fuchsin was tried, the color diffused into the medium around the red colonies, so that it was difficult, if not impossible, to recognize the typhoid colonies which may have lain in these areas. We have finally devised a medium that gives even better dif-

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ferentiation between the lactose-fermenting colonies and those that do not ferment lactose than the Endo plate and at the same time remains unchanged in the areas between the colonies. It consists of a combination of methylene blue and eosin in nutrient agar containing lactose and saccharose. On plates of this medium after 18 hours' incubation the colonies of typhoid are colorless and transparent, while those of B. coli are a deep black and do not transmit light. The medium immediately around the colonies of B. coli remains practically unchanged. Hence the plate is workable even when the colonies are close together. More of the feces, therefore, can be safely inoculated on this medium than on the Endo plate and it is to that extent more delicate and to be preferred.

The medium is prepared as follows: Nutrient agar is made in the usual way, containing 1.5% agar, 1% Witte's peptone, 0.5% sodium chlorid, and 0.5% Liebig's meat extract, to the liter of distilled water. It is cleared with egg-white, placed in flasks, and sterilized in the Arnold sterilizer on 3 successive days. The reaction is brought to +0.8. The agar is melted and saccharose (.5%) and lactose (.5%) are added. The medium is then heated for 10 minutes in the Arnold. To every 50 c.c. of the medium are added 1 c.c. of 2% yellowish eosin and 1 c.c. of 0.5% methylene blue. We always add the eosin first and then the methylene blue. The mixture is shaken and plates are poured. The surface of the medium is dried in the usual way before the plates are inoculated. We have also obtained excellent results by substituting for Liebig's extract, meat infusion rendered free from sugar by incubation with B. coli.

Stock solutions of 2% eosin and 0.5% methylene blue in distilled water are kept in the dark. We have not sterilized these solutions, as we found that they could be kept in the ice-box for weeks without causing contaminations of the medium. Ordinarily we do not heat the agar after the dyes are added, but we have demonstrated that the stained agar can be heated a half hour in the Arnold sterilizer without injury.

Eosin and methylene blue in distilled water in the proportion used, give precipitation; in the medium the agar acts as a "Schutzkolloid," preventing the formation of a precipitate.

The following experiment shows that methylene blue in even twice the amount contained in the medium as described, whether alone or in combination with eosin, does not inhibit the growth of the typhoid

bacillus. A strain of B. typhosus freshly isolated from the blood of a patient by culturing in bile was used. Two suspensions of the bacilli were prepared in salt solution and 1 loop of each suspension was inoculated on half of a plate, as shown in Table 1. The colonies were counted after 24 hours' incubation. They were of practically the same size on all the plates. The same nutrient agar with a reaction of + 0.7 was used throughout.

TABLE 1

Experiment Showing that Typhoid Bacilli are not Inhibited by Methylene Blue

Medium Used	Number of Colonies	
	Typhoid Suspension 1	Typhoid Suspension 2
Plain nutrient agar...	139	25
Agar + methylene blue (0.02%)................................	155	21
Agar + eosin (0.04%) + methylene blue (0.02%).............	135	26
Agar + eosin (0.04%)..	125	20

The following experiment was carried out to determine whether there is any inhibition of the typhoid bacillus when a typhoid stool is inoculated on the plate. A portion of the typhoid stool was rubbed up in salt solution and filtered first through a thin layer of absorbent cotton and then through filter paper. This filtrate was shaken and dilutions of 1:10 and 1:100 were prepared in salt solution. After this treatment it may be assumed that the typhoid bacilli present are distributed uniformly in the suspensions of the feces. One loop of each of the dilutions was inoculated on an Endo plate, on our methylene-blue eosin plate, and on a plain agar plate. The series of plates containing a convenient number of colonies was worked up in each instance with the results shown in Table 2.

Table 2 shows conclusively that typhoid bacilli in stools develop as readily on the methylene-blue eosin plate as on Endo plates or plain agar.

The chief advantage of this medium over the Endo plate, as already mentioned, consists in the fact that the colonies of B. coli become sharply differentiated from the typhoid bacilli without affecting the medium lying between the colonies, so that a typhoid colony can be readily recognized tho lying in close proximity to numbers of colonies of B. coli. There is of course a sharp limit to the amount of feces that can be safely inoculated on this medium, as is true of

every other medium. If the plate contains tens of thousands of colonies, each individual colony of B. coli remains very small and is poorly, if at all, differentiated from the typhoid colonies. It is certain, however, that this plate will stand a heavier inoculation than the Endo or the Conradi Drigalski plates.

The differentiation between the colonies of B. coli and those of the organisms that fail to ferment lactose is evident by reflected, as well as by transmitted, light; in selecting the suspicious colonies for fishing we use both, but rely chiefly on the picture afforded by the transmitted light.

TABLE 2

EXPERIMENT SHOWING THAT TYPHOID BACILLI INOCULATED FROM STOOLS ARE NOT INHIBITED BY METHYLENE BLUE

Patient	Medium	Total Number of Colonies	Number of Typhoid Colonies
J. L.	Endo	31	1
	Methylene-blue, eosin	42	3
	Plain agar	38	3
M. M.	Endo	29	15
	Methylene-blue, eosin	28	16
	Plain agar	37	22
M. O.	Endo	11	4
	Methylene-blue, eosin	12	4
	Plain agar	4	2
M. B.	Endo	44	21
	Methylene-blue, eosin	35	20

In addition to this main advantage the methylene-blue eosin plate possesses the following minor advantages:

1. The colonies of B. coli are differentiated earlier on this plate than on the Endo plate; that is, if the two plates are inoculated at the same time, the colonies of B. coli on the methylene-blue eosin plate in some instances will have taken on black centers while those on the Endo plate are still colorless or have merely a pink tinge.

2. A greater percentage of the colorless colonies turn out to be typhoid on the methylene-blue eosin plate than on the Endo plate. This is probably due in part to the fact that the former medium contains both lactose and saccharose, while the latter contains lactose alone, and in part to the fact that some of the organisms producing colorless colonies on the Endo plate fail to grow on the methylene-blue eosin plate.

3. There is complete inhibition of certain organisms which form small colonies on the Endo plates.

4. Certain bacteria which give colorless colonies on the Endo medium yield colonies with blue centers and transparent peripheries on our plate.

5. The Endo plate gradually turns pink on exposure to light; our plate remains unchanged. We have left it exposed to diffuse daylight for a period of 3 hours before inoculation without causing any noticeable deterioration.

6. The Endo agar is adjusted to $+ 0.2$, a reaction too alkaline to permit of the optimal growth of the typhoid bacillus. Our medium yields good results in agar, the reaction of which is best suited for typhoid. Slight variations in the reaction of the nutrient agar from which it is made, do not affect the efficacy of the methylene-blue eosin medium.

7. Since the medium around the colonies remains unchanged anyway, there is no reason for making the agar stiffer than it is in ordinary use.

Experiments were made also with the eosin methylene-blue combination of stains in nutrient agar with the addition of lactose, saccharose, and dextrose, and with the addition of dextrose alone, with a view to differentiating between typhoid and other non-lactose-fermenting colonies. There is no marked difference between the result given by the 3 sugars from that given by dextrose alone. On a plate containing the stains with 0.25% dextrose in agar, typhoid colonies grow well and are unmistakably differentiated from other colorless colonies when examined under the low power of the microscope, for they have peculiar blue centers which look like fine matted blue hairs. However, as this picture was not constant when typhoid stools were used, we decided to abandon the use of dextrose and depend on the colorless transparent colony on the eosin methylene-blue plate containing saccharose and lactose for diagnosis. If this plate be incubated for 48 hours, the typhoid colonies, even here, will often assume the centers described.

We have used this medium for the examination of scores of typhoid stools and mixtures of normal stools and typhoid cultures with very satisfactory results.

PLATE 20
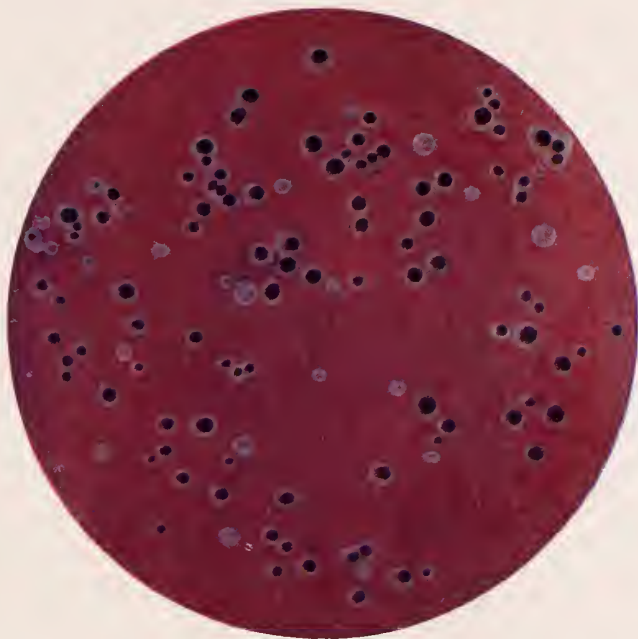
Reproduction of a methylene-blue eosin plate inoculated with a typhoid stool and incubated for 18 hours. The colonies of B. coli are black. The typhoid colonies are transparent. The largest transparent colonies are not typhoid.

A NEW DIFFFRENTIAL CULTURE MEDIUM FOR THE CHOLERA VIBRIO *

PLATE 21

OSCAR TEAGUE AND W. C. TRAVIS

From the Quarantine Laboratory, Health Officer's Department, the Port of New York

After it was found in this laboratory that a combination of eosin and methylene blue in certain definite proportions in lactose agar yielded an excellent plate medium for differentiating colonies of B. typhosus from those of B. coli,[1] experiments were planned to determine whether a similar differential medium for the cholera vibrio could be prepared. The cholera vibrio grew well on nutrient agar containing as much as 0.1% of eosin; but methylene blue and a number of other basic stains were found to inhibit the growth of the cholera vibrio to such an extent that their use for our purpose seemed out of the question. When the basic stains were used in combination with eosin, their toxicity for the cholera vibrio was much reduced, but not sufficiently to allow of their use in a strength approaching that in use in the typhoid medium.

However, bismarck brown proved to be far less toxic for the cholera vibrio than the other basic stains that we had previously used and hence our efforts were concentrated on it. The cholera colony was somewhat different in color from the colony of B. coli on nutrient agar containing eosin alone after 48 hours' incubation; the former had a red center, whereas the latter was uniformly pink. When a small amount of nutrose was added to the culture medium, the colonies were larger and this difference in color became apparent in 24 hours. Parallel tests were then carried out with yellowish eosin, bluish eosin, the pure French eosin, eosin A.G., and with erythrosin. The bluish eosin gave decidedly better differentiation than did the other stains.

On sugar-free nutrient agar to which were added 1% saccharose, 0.25% nutrose, and 0.0625% bluish eosin, the cholera colonies had red centers, while the colonies of B. coli were uniformly pink. We planted many mixtures of normal stools and cholera cultures on this medium and after 24 hours' incubation the cholera colonies could

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be distinguished at a glance from the other colonies, because the centers of the cholera colonies were several shades deeper in color than the other colonies and because the latter were of approximately the same depth of color at the center as near the periphery.

Bismarck brown was employed in combination with all the different eosins mentioned, and here, too, the bluish eosin yielded better results than the other eosins. Various proportions of the two stains were used, 0.0625% of eosin and 0.04% bismarck brown being finally selected as the optimum. The two strains added to nutrose saccharose agar in these proportions gave a medium on which the cholera colonies showed dark brown centers in 24 hours, while the colonies of B. coli were of a pale-pinkish or yellowish color (see Plate 21). The differentiation of the cholera colony was therefore much more striking than it was in the medium containing only eosin. When the cholera colony was buried beneath a large colon colony it might still be apparent as a minute black dot; on staining a smear from this dark area of the colony of B. coli, well-curved vibrios were seen. By transferring such material to peptone solution the cholera colony could readily be isolated from such a mixed colony in pure culture.

We prepare our medium in the following manner: Two pounds of chopped beef are soaked in 2 liters of distilled water in the ice-box overnight. The fluid is squeezed out, heated in the Arnold sterilizer, filtered through filter paper, made neutral to litmus by the addition of sodium hydrate solution, and again heated. After being allowed to cool it is inoculated with B. coli and incubated for 2 or 3 days. Nutrient agar is then prepared from it by adding 1% Witte's peptone, and 0.5% sodium chlorid, and clearing with egg in the usual way. The reaction is adjusted to —0.5. The nutrose (0.25%) is added after the agar has been cleared and filtered. A stock aqueous solution of bluish eosin (3%) is kept on hand in the dark. A 1% solution of bismarck brown in water containing 10% alcohol is also kept in stock. The bismarck brown is not completely soluble to 1% in distilled water alone.

To 50 c.c. of the nutrose agar are added 1% saccharose, 1 c.c. of 3% eosin solution, and 2 c.c. of 1% bismarck-brown solution. After this mixture has been shaken until the stains are uniformly distributed throughout the agar, plates are poured. Before being inoculated, the plates are uncovered and placed face down on the shelf of the incubator for 20 or 30 minutes to remove any excess of moisture.

Agar prepared with Liebig's meat extract, instead of the meat infusion rendered sugar-free by B. coli, yields similar results, but the differentiation of the vibrio colonies from the other colonies is not so good.

Usually we do not heat the medium after the stains have been added, but we have shown that such heating has no deleterious effect upon it.

When eosin and bismarck brown are added to distilled water in the proportions just mentioned, precipitation takes place; this never occurs however in the presence of the agar, the latter obviously acting as a "Schutzkolloid."

The dark center of the colony is due for the most part to the staining of the vibrios themselves, tho the medium immediately beneath the center of the colony is seen to be dark if the colony is scraped away.

Old laboratory strains of cholera differ greatly from freshly isolated ones in the size and appearance of their colonies on plain nutrient agar, in the degree of motility, in the rapidity of fermentation of sugars, and in a number of other respects. The results that we have obtained cannot be duplicated satisfactorily with old laboratory strains.

Our own strains have necessarily been grown on artificial culture media for several months; it is possible that strains fresh from cholera feces would give the same picture on bismarck-brown eosin agar without nutrose that our present strains yield on this medium with nutrose.

Through the kindness of Dr. Otto Schöbl we were in a position to plant 18 cholera-like vibrios on our medium. These vibrios had been isolated by him from cholera suspects or cholera contacts and resembled the cholera vibrio in almost all particulars, but they failed to agglutinate with anticholera serum. They all had a single flagellum, gave a positive indol reaction, and were hemolytic. Their colonies were found to resemble those of the true cholera vibrio very closely on our medium. This is perhaps an advantage rather than a disadvantage; for if cholera-like vibrios have been seen in the peptone it is well to be able to find them on the plates and show that they are not cholera vibrios.

We planted a large number of stools on Endo plates containing saccharose instead of lactose and then transplanted the red colonies

developing on these plates to agar slants. The saccharose-ferment-
ing organisms obtained in this manner were later inoculated on the
bismarck-brown eosin plate and in no instance did colonies resembling
those of the cholera vibrio occur.

This medium will have to be tried out in the isolation of vibrios
from actual cases of cholera and from convalescents from cholera
in order to determine whether or not it possesses practical value in the
diagnosis of cholera. It will probably prove to be much superior to
the plain agar plate but decidedly inferior to the Dieudonné medium.
In combination with a strongly alkaline peptone solution it may yield
excellent results.

After this work was completed Aronson[2] described a special
medium for the isolation of the cholera vibrio. It is prepared as fol-
lows: 35 gm. of agar are added to 1 liter of hydrant water and the
whole allowed to stand overnight. After the addition of 10 gm. of
meat extract, 10 gm. of Witte's peptone, and 5 gm. of sodium chlorid,
the mass is heated in a steam sterilizer at 100 C. for from 4 to 5 hours.
The flask containing the hot agar is then slanted to allow the coarser
particles to settle out and then the agar is poured into flasks gradu-
ated to receive 100 c.c. each.

The following stock solutions are prepared and heated one-half
hour in the steam sterilizer: (a) 10% solution of dry sodium carbon-
ate, (b) 20% solution of cane sugar, (c) 20% solution of dextrin.
A saturated alcoholic solution of basic fuchsin and a 10% solution
of sodium sulfite, the latter sterilized by being brought to the boil-
ing point, are also kept in stock.

To 100 c.c. of the agar are added 6 c.c. of the 10% sodium car-
bonate solution, and the containing flask is heated 15 minutes at 100 C.
The agar takes on a dark-brown color and becomes very cloudy.
While the agar is still hot, the following are added: 5 c.c. of the 20%
cane-sugar solution, 5 c.c. of the 20% dextrin solution, 0.4 c.c. of the
alcoholic-fuchsin solution, and 2 c.c. of the 10% sodium-sulfite solu-
tion. The flask is held in a slanting position until the coarser parti-
cles settle to the bottom; then plates are poured. The last of the
agar, containing the sediment, is discarded.

Cholera strains should yield large red colonies on this medium in
from 15 to 20 hours; at this time the colonies of B. coli are small
and colorless.

[2] Deutsch. med. Wchnschr., 1915, 42, p. 1027.

We prepared Aronson's medium according to these directions, but our strain of cholera which had now grown for several months on artificial culture medium, did not yield red colonies promptly. After adding 0.25% nutrose to the medium, however, we obtained remarkably good results with mixtures of our culture and normal stools.

We then substituted in this medium eosin and bismarck brown in the proportions recommended here for the fuchsin and sodium sulfite; similar results were obtained. The differentiation of the cholera colonies is as striking as on the eosin bismarck-brown agar of reaction —0.5 and far greater amounts of feces can be safely inoculated on the strongly alkaline plates.

The colonies of B. coli on the Aronson medium become red on further incubation, obscuring the cholera colonies. On the eosin bismarck-brown strongly alkaline agar the colonies of B. coli remain pale after 48 hours' incubation and the cholera colonies are much darker than after 24 hours' incubation; hence the cholera colonies, instead of becoming obscured, are even more sharply differentiated from the other colonies upon further incubation.

Finally, we substituted for the fuchsin and sodium sulfite in the Aronson medium plus nutrose, 4 c.c. of 3% bluish eosin and obtained excellent results with this medium also. The cholera colonies were colored deep-red while the colonies of B. coli were pink.

Aronson's medium and our two modifications of it give results very similar to what one is accustomed to get with the Dieudonné medium. Which of these four kinds of media is best can only be determined by extensive parallel tests on the stools of convalescents from cholera. The first three media possess the advantage over the Dieudonné medium of being always ready for immediate use.

PLATE 21
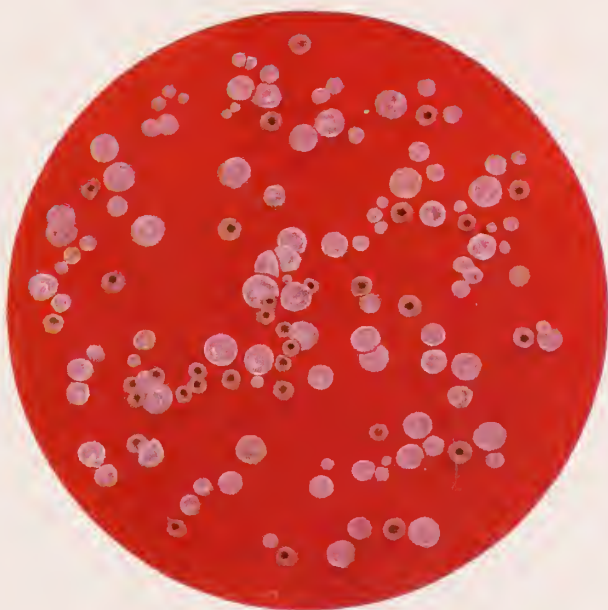
Reproduction of a bismarck-brown eosin plate inoculated with a mixture of a normal stool and cholera vibrios. The cholera colonies have dark-brown centers.

STUDIES ON DIPHTHERIA IN CLEVELAND, I *

DIPHTHERIA-CARRIERS

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INTRODUCTION

This paper forms the beginning of a series of studies of diphtheria in Cleveland with special reference to the problem of shortening the average quarantine period as far as is consonant with the safety of the public. The material available is perhaps of unusual character, inasmuch as it represents the combined material of the division of health, the contagious department of the City hospital, and the various infant asylums and other similar institutions in and about Cleveland. Through the co-operation of the municipal authorities, the Western Reserve medical school, and certain of the private institutions, it has been possible to gain access wherever desired, and the development of these opportunities gives promise of an even more abundant material in the future.

The matter is being taken up from a variety of standpoints, among which the morphologic and immunologic are prominent in the present series. From year to year the statistics of diphtheria in Cleveland are being worked out under the direction of one of us by students in the medical school of Western Reserve University, as theses for the course in hygiene, and published in the Cleveland Medical Journal. When the number of cases recorded and analyzed is sufficient, the statistical side will be considered.

We desire to thank the division of health for assistance in the use of the records, and the various institutions for the courtesy with which they have met our desires and suggestions.

THE INCIDENCE OF DIPHTHERIA-CARRIERS IN CLEVELAND

The problem of diphtheria-carriers is one of the most important in the management of this disease, for it is probable that the spread and continuance of the epidemics are due largely to the presence of diph-

theria bacilli in the throats of apparently healthy persons. The litera-
ture at present is so filled with articles on the number of such persons
and on the results of various attempts to get rid of the organisms
that one hesitates to add further articles to the array. The fact,
however, that we have been able to combine in Cleveland a number
of series of cultures from a variety of sources with some degree
of careful investigation into the various groups seems to offer justifica-
tion for such an addition.

Definition of Carrier.—In order that our investigations and our
results may be clearly interpreted, certain facts must be set down.
The first of these has to do with the question: What is a diphtheria-
carrier? A diphtheria-carrier is a person free from clinical symptoms
of diphtheria, who carries virulent B. diphtheriae in the nose or
throat for a period of more than 21 days.

The significant word in this definition is "virulent," and the question
of virulence is our chief stumbling block. We note in our series that
there are: (1) organisms morphologically and culturally B. diphtheriae,
but incapable of producing diphtheria toxin, occurring in all groups;
and (2) organisms of a similar morphologic type, occurring in large
numbers in institutions where detailed cultural investigations are not
practicable. In these institutions there has been no epidemic of diph-
theria, and indeed no cases have come under treatment for a period
of years, these facts suggesting that the virulence must be compara-
tively low.

Moreover, we feel that tho inoculation of guinea-pigs with fatal
results and recovery of the organism is diagnostic of virulence, yet it
does not follow that organisms failing to cause the death of guinea-pigs
are nonvirulent for man, tho the probability of this is strong.

In general, then, an organism isolated from nose or throat, and
morphologically identical with known virulent types, may be virulent
or avirulent. Establishment of cultural reactions identical with those
of the virulent type still offers no further proof. Only where animal
inoculations are possible can the determination of virulence be made.
The recent work of Zingher and his co-workers[2] simplifies this to
a large extent but still leaves the condition of the poorly financed labora-
tory unsatisfactory, so that while some cases may be released while
still dangerous, more will be quarantined beyond the necessary period.

In addition to virulence, we must establish the date after diagnosis
on which the carrier state may be said to begin. This must of necessity

be arbitrary, as no two cases become free after exactly the same interval. In the tabulation of our results we found that the average duration of quarantine, estimated from the records of the bureau of laboratories for 1913, 1914, and 1915, was 20.5 days, so that an arbitrary basis of 21 days was established. This calculation is based of course on those cases only in which a diagnosis of diphtheria had been made, and in which therefore we may infer the persistence, after convalescence, of a virulent organism. It gives us no information as to the carriers with no history of infection, in whom the presence of the organisms is established accidentally. The Schick test is helpful in that it has been established that all carriers must be negative to this test; but of course at present only a very small percentage of any community has been tested.

On the basis of the evidence available in Cleveland to the present time, we must consider as carriers all in whom morphologic B. diphtheriae persists beyond 21 days.

Analysis of Cleveland Series.—Various attempts to ascertain the average number of diphtheria-carriers in a given population have shown a surprisingly large number, and one of the purposes leading to the preparation of this paper was to establish a similar average for Cleveland. To the routine cases coming to the laboratory for diagnosis, we have added certain groups of persons who were examined with the sole view of establishing the presence or absence of B. diphtheriae or of other types. As will be noted in the detailed morphologic investigation, we have taken certain standards as representing certain types, and it is on these standards that our figures are based. The investigation covered the following groups:

A. Public Schools.—Of 1,024 children in grade schools scattered over the city, ranging in age from 9 to 12 years (the children of a given grade in each school being examined on the same day), 42, or 4.2%, showed morphologic B. diphtheriae.

B. Orphan Asylum.—This group is of special interest inasmuch as for 6 years there has been no diphtheria reported from the institution, nor has any antitoxin been given. The cultures were originally taken in the hope of establishing more data as to the throat flora under such conditions, but the results in this connection must be taken cautiously, as the children have free intercourse with the street children of a crowded district. Moreover, several cases of tonsillitis and sore throat have been treated each year. The records of the hospital ward, how-

ever, show no serious cases of illness in any way attributable to diph-
theria, nor have there been any after effects suggestive of diphtheria
paralysis. This would indicate that, if any of the cases of tonsillitis
were diphtheria, they must have been mild. Moreover, in such cases
as were tested on animals no virulence was found.

Of 429 children in this asylum, in ages ranging from 4 to 15, with
separate cultures from nose and throat in each case, or 858 cultures in
all, 46 showed morphologic B. diphtheriae in the throat alone, 2 in the
nose alone, and 2 in both. This gives 11.2% carrying morphologic B.
diphtheriae.

In connection with an epidemic of cerebrospinal meningitis a year
later, cultures were made from all the inmates of this same
asylum. In 11 of 535 persons morphologic B. diphtheriae was found,
the results showing just over 2% carriers as against 11% one year pre-
viously.

C. Babies' Dispensary.—Of 558 routine cases coming to the Babies'
dispensary, 3 showed morphologic B. diphtheriae, and the cultures in
these cases were fatal for guinea-pigs. The patients in this series being
all under 3 years of age, the contact with other children was more
limited than in the other groups. The percentage of 0.54 showing
morphologic B. diphtheriae was markedly lower than that of other
groups, but all found were virulent.

D. Boys' Reform School.—Here, among boys from 6 to 18, two
sets of cultures were made at a 5 or 6 weeks' interval in relation to an
outbreak of diphtheria. The farm is in the country some miles from
Cleveland, so that contacts can be accurately determined. The apparent
etiology of the epidemic was a new boy who ran away after 48 hours'
detention and later turned up in Cleveland with a clinical case of diph-
theria, from which at least one contact case developed, showing the
virulence of the organism. Three days after his departure from the
farm the first case developed there. The culture was sent in to the
laboratory of the division of health, and some of the staff were detailed
to make cultures and Schick tests on all inmates, except those suffering
from clinical diphtheria. Of the cultures from 175 boys, there were 7
in the first set and 8 in the later set, or 4% and 4.5%, that yielded
morphologic B. diphtheriae.

All the boys who gave positive Schick tests were given antitoxin
and no further cases developed.

E. Infants' Asylum.—Of 53 cultures from children ranging in age
up to 5 years, none showed morphologic B. diphtheriae. Our record

of a second trial at this asylum is omitted, as the interval was only a few weeks, and the results identical with those of the first.

This gives a total of 2,774 persons examined in special investigations in and about Cleveland, among whom we obtained 105 cultures of morphologic B. diphtheriae, a percentage of 4.07. The results of Moss[6] in Baltimore showed a percentage of 3.5.

Routine Diagnosis Series.—The cases sent to the city laboratory for diagnosis were clinically sore throat, and were interesting from the carrier standpoint in regard to the length of quarantine, giving an index of the time during which convalescence may be dangerous.

Period of Quarantine.—During the last 3 years careful records have been made of the length of quarantine in these cases. In 1913 there were 2,067 houses quarantined, in 1914 there were 1,795 houses and during the first 10 months of 1915 there were 981 houses. This excludes all from which the patients were transferred to the city hospital prior to the last part of 1914, as their cultures up to that time did not come to us for release, and excludes also all in which cases terminated fatally. A small error must be considered here inasmuch as the length of the quarantine period was taken as the length of the quarantine of the house, so that when several cases followed one another in the same family, the quarantine was not raised until the last case showed 2 negative tests. The proportion of such cases, however, was small, and the labor of following out the individual details would have been excessive.

TABLE 1
Period of Quarantine

Days	1913	1914	1915 (10 mo.)
3 to 10...........................	404	125	118
10 to 20...........................	731	968	490
20 to 30...........................	401	426	240
30 to 40...........................	240	146	87
40 to 50...........................	124	48	26
50 to 60...........................	61	14	13
60 to 70...........................	35	8	6
70 to 80...........................	27	4	1
80 to 90...........................	23	3	...
Over 90...........................	21	3	...
Total quarantine................	2067	1745	981
Average number of days.......	22.9	19.7	18.9

Total houses quarantined 4843; average quarantine for the 3 years 20.5 days.

The table is inserted here in the same form as for the annual reports of the health division. The summarization shows that over one-half the

quarantines terminated before the end of the 3rd week, but that in a comparatively small number of cases organisms persisted for a period measured in months.

MORPHOLOGIC STUDIES

Much time has been spent on this phase of the question, but the difficulties entailed in the study of an organism with such a tendency to mutation are so great, and the use of terms in current literature is so confusing, that investigations have been undertaken from a different point of view, which will be presented in a subsequent paper. On account of this notable confusion in the literature, it appears necessary to establish clearly our own definitions, so that when we speak of diphtheria, pseudodiphtheria, and so forth, our meaning, at least, may be clear.

Terminology.—The use of the words "type" and "form" as interchangeable terms appears to us unjustifiable. A type must remain constant, and may be the precursor or the basis of a variety of forms. In our experience a culture containing only one type is very rare, if indeed it exists, and careful examination will usually show several types under the Wesbrook classification. With possibly rare exceptions, we consider B. diphtheriae in 15- to 24-hour cultures as an organism of rod form staining irregularly and containing metachromatic granules. Our commonest form shows two lightly staining areas adjacent to the granules, leaving a cleft in the center. Marked variations occur with alterations in the source, in the reaction and moisture of the culture medium, and even in the method of its sterilization. That a change in the chemical or biologic conditions of the throat may also cause variations is suggested by the fact that the diagnostician can often diagnose a culture as "for release" by morphology alone.

Diphtheria-like Organisms.—Most prominent is the Hofman-Wellenhof organism. As to the virulence of different forms of diphtheria bacilli, it is surprising, after the large amount of investigation, to note the great variance of opinions even at the present day, a matter of some importance when one laboratory contradicts the opinion of another as to a diagnosis culture. In view of the work in hand and of the large number of articles on the subject already in print, we shall not attempt here to analyze the opinions on this point, further than to summarize.

Some authors hold that the Hofman or D_2 type is at times pathogenic, and have claimed mutation between this type and the typical one

in both directions; others believe that the phenomenon of virulence or avirulence is a permanent one; others report virulent pseudodiphtheria not affected by diphtheria antitoxin. In this laboratory, while we hold sub judice the statements about virulent organisms of this type, we feel that until these organisms are proved contagious in the sense that the Klebs-Loeffler bacillus is contagious, we may safely consider them as negative, and refuse to quarantine patients on their evidence. In our own experience organisms of this type have never proved virulent.

TABLE 2

MORPHOLOGY AND VIRULENCE

Source	Number of Cases	Morphologic B. Diphtheriae	Virulence	Diphtheria-like Bacilli, inc. Hofman
A. Babies' dispensary	558	3	3	175
B. Boys' reform school	175	5	..	82
C. Orphan asylum, 1914	429	46	*	132
D. Orphan asylum, 1915	535	11	..	173
E. Public schools	1024	42	..	106
E. Infants' asylum	53	0	..	23
F. Routine diagnoses	....	..	..	...
Total	2774	110	3	691

* 18 tested; none virulent.

In the interpretation of Table 2, certain points are conspicuous:

1. The large percentage of diphtheria-like organisms, more especially with respect to babies and children (A and E)—32%. Fourteen cultures were selected for further investigation; all proved nonvirulent, and nonproductive of acid in dextrose, dextrin, and saccharose. One even gave an alkaline reaction in the sugars.

2. The large percentage in the orphan asylum—37% in 1914 and 32% in 1915. In this institution, as noted elsewhere, there has been no clinical diphtheria for 6 years. Eighteen of the diphtheria-like organisms were nonvirulent and nonproductive of acid in dextrose and dextrin.

For our present purposes we have accordingly divided the organisms into 3 groups, while appreciating that the variations already noted as due to environmental factors may have led us astray at times: (1) virulent B. diphtheriae, (2) avirulent B. diphtheriae, (3) diphtheria-like organisms, including diphtheroids.

Group 1 includes irregularly staining organisms with terminal granules (including both C and D types and involution forms such as *A and B). There is a tendency to parallelism. These organisms produce

acid in dextrose and dextrin, but not in saccharose, and in 48-hour broth cultures are fatal to guinea-pigs in doses of 0.5% of body weight. Organisms less than 12 hours old show the usual solid forms similar to the Hofman bacillus.

Group 2 includes forms morphologically and culturally identical with those of Group 1, but not productive of toxin.

Group 3 includes several subgroups. The chief forms are those similar to Wesbrook's D_2, E_2, and G_2 with lanceolate or rounded ends, varying markedly in length. These include the Hofmann bacilli. In older cultures they may become so short as to resemble diplococci, but occasionally aging produces long forms similar to some of the involution forms of Groups 1 and 2. At from 36 to 48 hours terminal granules often develop, clouding the picture still more. Parallelism is even more marked than in Groups 1 and 2, but no acid is produced in dextrose and dextrin. In our experience these organisms are never virulent. Another subgroup is represented by B. xerosis. This is a short solid-staining form in the early cultures, becoming barred and even granular on further incubation. It never produces gas in dextrose and dextrin, but may in saccharose. It does not produce toxin. Finally, there are the so-called "diphtheroids," found in a variety of pathologic conditions, and of disputed virulence. They are readily distinguishable from diphtheria bacilli, and in addition are rarely found in the throat. There is another group of rod forms with metachromatic granules, often found in the throat and occasionally mistaken for diphtheria bacilli. These are of B. lactimorbi type, and, by the experienced worker, are easily differentiated by their more regular morphology. They form spores and are motile.

In view of our present knowledge we believe that the most satisfactory attitude in regard to diphtheria, is that which eliminates all diphtheria-like forms as etiologic factors in the disease. In methods and principles of diagnosis, there should be a greater uniformity, so that the results of one laboratory can be properly interpreted at another.

IMMUNOLOGIC STUDIES—THE SCHICK TEST

As an aid in the study of diphtheria-carriers, we decided in the early part of 1914 to make use of the so-called Schick, or intradermal, test for antitoxin in the blood. Through the kindness of Dr. W. H. Park, of the New York city research laboratories, arrangements were made enabling us to get a fresh supply of diphtheria toxin every 2 weeks.

In the beginning of the work it was deemed necessary to confirm the data as to percentages of positive and negative reactions at various ages, to determine the possibility of error in making the test and reading the reactions, and to determine the action of the test in the study of carriers.

The material was obtained from the following sources:

A. Boys' farm 175
B. City hospital. Selected cases........................ 240
C. City hospital. Routine cases........................ 232
D. Infant asylum 53
E. Laboratory staff (Repeated tests showed same results
     as original) 20
F. Medical students 38
G. Dental students 30
H. Miscellaneous 38
                                                      ———
                                                      826

In articles by Schick,[1] Park, Zingher, and Serota,[2] and later articles by Kolmer and Moshage,[3] and Bundesen,[4] the percentages of positive reactions found at different ages have been given. To these we add our own, which we find are quite in agreement with their findings (Table 3). With slight differences it will be seen that about 80% of infants under 1 year, from 50 to 60% of children over 10 years, and about 70% of adults have enough antitoxin in their blood to render them insusceptible to diphtheritic infection and to make unnecessary the prophylactic administration of diphtheria antitoxin if they have been exposed to the disease. Schick[5] has recently touched on this phase of the subject.

The technic for making the test has been repeatedly described, but we wish to emphasize that altho it is very easy to perform, slight errors may cause false reactions which are not easily differentiated from true reactions. The false reactions fall into several groups:

1. Those resulting from a larger amount of fluid than necessary. We agree with Kolmer and Moshage that the smaller amounts, from 0.05 to 0.1 c.c., are much better. The injection of 0.2 c.c. not infrequently gives rise to a traumatic reaction which might confuse the inexperienced person.

[1] München. med. Wchnschr., 1913, 60, p. 2608.
[2] Arch. Pediat., 1914, 31, p. 481.
[3] Am. Jour. Dis. Child., 1915, 9, p. 189.
[4] Jour. Am. Med. Assn., 1915, 64, p. 1203.
[5] Ztschr. f. d. ges. exper. Med., 1914, 2, p. 83.

2. Those resulting from trauma due to using a large needle. We have found most satisfactory the platino-iridium No. 25. If the needle is introduced with the lumen directed upward, the white blister-like swelling will be obtained at every trial. We have never observed a false reaction from trauma persist for over 24 hours.

3. Those resulting from anaphylaxis of a general protein character. These reactions are rare, and when occurring rarely persist beyond 24 hours. Therefore by reading the reaction at the end of 48 hours most of them are eliminated.

4. Those resulting from the preservative added to the toxin solution injected. Kolmer and Moshage have noted this reaction in 46% of scarlet fever patients when 0.5 c.c. of sterile salt solution containing 0.25% of tricresol was injected intradermally.

We have found that the use of freshly sterilized salt solution gives a considerably lower number of false reactions than when old salt solution is used. The reactions obtained with old, while not as marked as the other-mentioned false reactions, still might confuse inexperienced persons.

TABLE 3

Percentages of Persons Susceptible to Diphtheria According to Age

Age in Years	Schick	Park	Kolmer	Bundesen	Perkins, Miller, Ruh	Average of Reports
Under 1	7	40.0	12.0	24.5	15.2	19.7
1 to 5	53	65.9	54.5	51.5	58.1	56.6
5 to 10	50	42.5	57.5	36.7	51.6	47.7
10 to 15	50	26.0	24.0	42.2	34.4	35.3
Over 15	10	33.0	35.0	33.0	31.0	28.4

An additional carefully checked series of 41 medical students and 28 dental students gave a surprisingly high percentage of positives, practically identical in both groups. There were in all 48 positive tests, 4 of which did not develop until the afternoon of the second day, and none of which failed to persist more than 4 days, and 21 negatives in which no reaction took place. This gives 69% of persons susceptible. Further tests, which will involve the entire personnel of the medical school, are now in progress to determine whether this high percentage was accidental or characteristic of the group.

Concerning the practical use to which the test can be put in the study of diphtheria-carriers, we believe that it offers help in making the diagnosis of a true carrier. Guthrie, Moss, and Akers,[6] in a study

[6] Bull. Johns Hopkins Hosp., 1915, 26, p. 88.

of the incidence of diphtheria-carriers, including over 6,000 adults, found a striking discrepancy between the number of carriers and the incidence of clinical diphtheria. In their study they find that 80 to 90% of the organisms isolated were avirulent. In our own studies similar results were obtained for those organisms tested as to virulence. Thus, in these cases in which an organism morphologically a diphtheria bacillus was found, the person would necessarily be placed in quarantine. If an intradermal test gave a positive result, the patient could be discharged without further investigation of virulence. If, however, a negative reaction was obtained, a virulence test would have to be made.

In our series of cases 16 carriers, according to our definition, have been subjected to Schick tests and no positive reaction has been found. This confirms the well-known fact that true carriers invariably have an antitoxin content of over 1/30 unit per cubic centimeter of blood.

In the epidemic at the Boys' farm elsewhere described, Schick tests made in the case of 175 inmates, gave positive results in 16, and a prophylactic dose of antitoxin was given each of these. No isolations were carried out other than those of the clinical cases and there were no additional infections. In a large infant asylum, of 53 children tested, 6 were positive, all of which were treated by Behring's method for active immunization. Altho only 3 became negative to the Schick test after this treatment, no further cases developed in the asylum.

The test is now being made on all the students in Western Reserve University Medical School, to establish the mode of procedure in case of an epidemic.

A VARIATION OF GEMMATION OF BLASTOMYCES DERMATITIDIS IN THE TISSUE LESION *

PLATES 22 AND 23

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While a series of cases of systemic blastomycosis was being studied microscopically, there was encountered in a skin lesion of one of these a condition so unusual and at first so difficult of explanation that it was made the subject of a particularly careful morphologic study. Tho there were numerous budding organisms typical of the so-called blastomyces, the presence of numerous minute forms at first suggested sporulation. This peculiarity may be emphasized by a brief description of the typical appearances of the parasites found in the lesions of the recognized yeast-mold infections.

Tho originally recovered from the more common skin affection and named accordingly, Blastomyces dermatitidis (Gilchrist and Stokes[1]) is the cause of systemic blastomycosis. It appears in the tissues as spherical, ovoid, or irregular bodies possessing doubly contoured highly refractile cell membranes, which vary considerably in thickness in different individuals. The parasites show considerable variation in size, the average being from 12 to 20 microns in diameter. The protoplasm is irregularly granular, at times vacuolated, and is as a rule acidophilic, tho the contained granules often stain with the basic dyes. These granules are apparently not connected with spore-formation, and this parasite is held to multiply in the tissues only by budding. During the process of formation and subsequently the buds are seldom less than from 4 to 6 microns in diameter, and can be clearly made out with the ordinary dry lenses. Coccidioides immitis (Rixford and Gilchrist[2]), the causative agent of coccidioidal granuloma, has been shown to be a distinct type of fungus, most recently by McNeal and Taylor[3] and by Brown and Cummings.[4] Altho in the tissue lesions it resembles the blastomyces somewhat, it usually appears larger and less hard-walled. The individuals vary more widely in size, ranging from 5 to 50 microns in diameter. The protoplasm may be finely granular or vacuolated, or may be segmented into numerous irregular daughter cells. Other large bodies are filled with numerous smaller spheres or spores, usually flattened where apposed, and each surrounded by its individual capsule. Large ruptured capsules are commonly found in the lesions but it is said that the small

[1] Jour. Exper. Med., 1891, 3, p. 53.
[2] Bull. Johns Hopkins Hosp., 1896, 1, p. 209.
[3] Jour. Med. Research, 1914, 30, p. 261.
[4] Arch. Int. Med., 1915, 15, p. 608.

parasites, either naked or encapsulated, are not recognizable ordinarily until they have enlarged somewhat and the distinguishing capsule has developed. Multiplication by budding is said never to occur in this organism, and it is now generally accepted as a species distinct from the blastomyces.

In but few reports in recent literature was it thought that indications of both budding and sporulation of the parasites had occurred in the tissue lesions or exudates. LeCount and Myers[5] studied microscopically and carefully described the lesions in a case of systemic blastomycosis originally reported by Eisendrath and Ormsby.[6] Widespread lesions were found, in all but one of which the organisms were of the ordinary type. In the cerebellum, however, was an area in which were parasites thought to have multiplied by endosporulation. Very minute blastomycetes occurred in dense clusters numbering several hundreds. Among these there were a number of budding cells, but not enough to account for the great numbers of minute forms. In some of the aggregations large torn capsules were found, but it was said that intermediate forms of development between the supposed spores and the mature organisms were not demonstrated.

Montgomery[7] described a case which, while not identical with that recounted, is at least similar in that the organism present was thought to multiply both by budding and by spore-formation. In nearly every tube inoculated from unbroken abscesses pure cultures of the blastomyces were obtained, and the organism was unusually pathogenic for guinea-pigs. Nevertheless, in the smears and sections from both man and animal, there were found exceedingly few organisms in the ordinary forms. There were, on the other hand, large numbers and masses of round cells, each about the size of a red blood corpuscle, strongly resembling small blastomycetes but without the usual double-contoured capsule. No large sporulating forms could be found. Montgomery suggested in this publication that these were possibly young rapidly multiplying organisms, tho no relation could be traced between the small bodies and the larger budding forms occasionally met with. In his publication with Ormsby,[8] however, this case was summarized and the statement made that these organisms were thought possibly to have been produced by a process of spore-formation.

In several other instances unusual variations in the morphology of the blastomyces are described, but not completely enough positively to establish distinct type variations in the infecting organisms. Hektoen[9] described very minute forms which developed into the larger. Ricketts[10] mentions finding in a teased specimen of excised tissue a small form, found together with the typical organisms. These smaller bodies occurred in pairs and chains, were encapsulated, and measured about 3.75 microns in diameter. Their nature and significance were not discussed. Another inconclusive, but very interesting, description was made by A. J. Smith[11] of a small type of organism which by special methods stained differentially both from the ordinary blastomyces and from body cells. They were round to oval, with thick, single, deeply staining basophilic capsules and lightly basophilic centers, the latter containing several vacuoles suggesting endogenous spores of yeasts. Because of lack of fresh material, these organisms could not be studied culturally. In

[5] Jour. Infect. Dis., 1907, 4, p. 187. Tr. Chicago Path. Soc., 1907-8, 7, p. 49.
[6] Jour. Am. Med. Assn., 1905, 45, p. 1045.
[7] Jour. Cutan. Dis., 1907, 25, p. 393.
[8] Arch. Int. Med., 1908, 2, p. 1.
[9] Jour. Exper. Med., 1899, 4, p. 261.
[10] Quoted by Montgomery, Jour. Cutan. Dis., 1901, 19, p. 38.
[11] Med. Bull. Univ. of Pennsylvania, 1909-10, 22, p. 362.

a case reported by Hildreth and Sutton[12] from Porto Rico, there were observed beside numerous typical cells, many of which were budding, numerous smaller spherical bodies about 3 microns in diameter. No further description of these is made.

In the skin lesions of a case which will be reported more fully elsewhere has been found a condition more particularly like that described by LeCount and Myer, in which there were vast numbers of minute parasites very suggestive of spores. Study of this lesion in specially stained preparations demonstrates a condition of the organisms present which has not, so far as can be learned, heretofore been accurately described.

The essential features of the case may be summarized as follows: A colored man, 18 years of age, presented himself at the hospital with a dermatitis, thought to be pellagrous, over hands, wrists, neck, and ankles. In subcutaneous tissues of the inguinal region there was an added infiltrative lesion of tubercular type. Over this the epithelium was thickened and rough and showed numerous small fissures covered by encrusted exudate. In places a yellowish purulent material could be expressed from the deeper tissues. Tubercle bacilli were found in the sputum. At autopsy (Dr. T. D. Hurley) lesions typical of tuberculosis were found in the lungs. Sections of the lung tissue showed extensive granulomatous lesions with necrosis, in many of which tubercle bacilli were demonstrated. Here and there, however, a number of typical encapsulated blastomycetes occurred. A few were lying free but the majority were intracellular. All were mature thick-walled forms, very few of which showed evidence of budding. No other deep organ was found to be involved in either the tuberculous or the blastomycotic process.

Skin Lesion.—In sections of skin taken from the inguinal region there was a condition which superficially resembled the lesion of coccidioidal granuloma as well as that of blastomycosis. The epidermis showed little of the usual downward proliferation and the parasites were for the most part in the deeper layers. In the firm connective tissue were lesions which appeared to be abscesses, immediately about which the chorium was partially degenerated and more or less infiltrated with lymphoid and plasma cells. Few polymorphonuclear leukocytes were found. Within such an area and merging into the central region was a zone which was of unusual type. This area at first glance was suggestive of ordinary necrosis, was seen to be made up very largely of rather unusual "endothelioid" cells (endothelial leukocytes) which had for the most part fused to form great numbers of giant cells (Fig. 1). These giant cells presented an unusual appearance; they were often very large and presented coarsely granular appearance (Fig. 2). So numerous were they that the central broken down material seemed often to have been derived entirely from their disintegration. This process was not entirely an infiltration by endothelial leukocytes, but was also degenerative. This was shown not only by the general appearance of the affected skin, but by the fact that in the lesions wellformed blood-vascular spaces of fair size were seen at times quite unsupported by other than the described cells of the granuloma (Fig. 1). These vessels

[12] Jour. Am. Med. Assn., 1914, 63, p. 2289.

were not at all the new vessels of granulation tissue. A feature of the lesion was that, notwithstanding the amount of endothelial-cell infiltration, giant-cell-formation, and necrosis together with, as will be described, the presence of vast numbers of blastomycetes, there was often practically no fixed-tissue reaction or invasion by leukocytes of the ordinary types. Where such infiltration was present, it was not commensurate in extent with the lesion.

Scattered throughout this lesion, among the cells and cell debris but particularly within the giant cells, were great numbers of blastomycetes of all sizes and of varying appearance (Figs. 3, 4, and 5). Numbers of these were in the common spherical and budding forms of the organism. Beside these, however, there were seen, on close scrutiny, vast numbers of organisms so small and so massed as to give to the giant cells and necrotic material their coarsely granular appearance. This is seen particularly well in Fig. 2. Very many of these minute organisms measured about 1.5 to 3 or 4 microns in diameter and frequently could be defined only as large granules, many of which were indistinguishable from the protoplasmic granules of the surrounding structures. Only the larger of these small organisms could clearly be seen, in ordinarily stained specimens, to possess capsular membranes. In the outer zones of a focus of infection this minute form of the organism was usually absent, or present in small numbers. Nowhere were tubercle bacilli demonstrable in specially stained sections.

Altho the parasites differed widely in appearance from those found in the tissues of the coccidioidal granuloma, it was thought that the small forms were produced by some process of spore-formation. and careful search was made for ascospores. Occasional broken shell-like structures similar to those described by LeCount and Myer[5] could be found, some of which contained more or less numerous minute parasites, but no organism was encountered which showed any evidence of endosporulation.

An occasional mature cell was found to be divided into two distinct individuals within the sclerotic outer shell by a transverse wall of the more delicate inner or soft membranous capsule. These daughter cells did not resemble spores, and in no instance was this process seen to further subdivide the organism. From the finding in the special preparations later to be described of two or three organisms which showed incomplete stages of this subdivision, it was concluded that these forms resulted from an attempt at ordinary budding which was restrained by the inability of the primary pseudopodium of the bud to rupture the sclerotic shell. This process may be described as originating by a herniation of the inner capsule at one point, the resulting "pseudopodium" being forced to occupy a position between the mother cell and the hard capsule. This process seems not to have been mentioned in previous descriptions of the organism.

Comparison of Staining Methods

In order to determine the best staining technic for the demonstration of the minute organisms in this lesion, as well as for routine use in the study of blastomycotic lesions, a great many preparations were made, and tissues from several cases subjected to many special processes. This work was made necessary by the fact that few of the recent authors on blastomycosis discuss such special stains at any length, and the processes referred to in the literature available at the time, proved inadequate for the purpose. The results obtained will be indicated briefly.

Tho recommended by some, the modifications of Romanowski's stain were employed with but indifferent success. The best of these was Giemsa's. Altho it has been said to stain the capsule a faint blue, in my hands it left both Zenker's and formalin-fixed tissues uncolored. The cytoplasm usually assumes a blue or purplish color which is but slightly different from that of the cell nuclei. Wright's and Leishman's stains were less satisfactory. Even the eosin and methylene-blue technic of Mallory, tho it sometimes gives very pretty results and is said by Wolbach[13] to be the best for demonstrating the coccidioides in tissues, failed to give clearly differential results in the special lesion studied. Unna's polychrome methylene blue was similarly disappointing.

In view of the usefulness of allied processes in the study of smear preparations of certain yeast organisms, as described by Verity[14] and others, it had been hoped that Levaditi's silver-impregnation method, or some modification of this, would prove differential for the capsules of the blastomycetes. In the tissues available, however, which had been in formalin for some time, this method was also without value. It was also expected that at least certain forms of the blastomycetes would exhibit affinity for stains used in demonstrating amebae in the tissues. This proved not to be the case. It may here be added that the results with Van Gieson's stain and with Mallory's phosphotungstic-acid hematoxylin stain were similarly unsatisfactory, tho the protoplasmic granules were made very prominent by the latter method, as well as by the ferric-chlorid and hematoxylin method. Bacterial stains for tissue sections in which the principles of Gram's stain are used failed to stain the blastomycetes, tho bacteria were well demonstrated. Solutions of thionin (including carbol thionin) and of methylene blue are often useful for the study of the details of the cytoplasm of the organisms, particularly in the study of exudates, etc., in moist or fixed preparations. In sections of fixed tissues, however, they are not satisfactory.

Carbolfuchsin staining, when the stronger acids were used in the decolorization, was productive of nothing worthy of note, all structures giving up the primary stain. When, however, the differentiation was carried out with very weak acids or with plain alcohol, a curious result was observed in the sections of the skin lesion under particular consideration. These were counterstained with alcoholic methylene blue and directly cleared with xylol. Among the great numbers of blue-stained parasites there was seen here and there an individual made very prominent by its intense bright-red color. This was due entirely to retention of the primary stain by the protoplasm, and under no circumstance had the cell-membrane itself shown evidence of acidfastness. These acidfast bodies varied considerably in size and apparent maturity and showed nothing which might suggest an explanation of this peculiar staining reaction.

13 Jour. Med. Research, 1904, 13, p. 53
14 Lo Sperimentale, 1912, 66, p. 1.

There may be an analogy between this observation and that of Wolbach[13] that in the coccidioidal granuloma an occasional sphere retains Scharlach R.

The anilin-blue connective-tissue stain of Mallory was the staining principle which, of all those tried, gave the most constant and striking results in the demonstration of the blastomycetes in general. Modified as to length of application, it was the only stain which clearly demonstrated the parasites to be described.

Since this work was done, a study of the organisms from a case of blastomycotic dermatitidis by Bowen and Wolbach[15] has been made accessible. These authors, tho they do not define the exact technic used, also concluded that the connective-tissue stain was the most valuable for the demonstration of the organisms. Hektoen[16] speaks of its use by Wolbach. The only other report in which its use is indicated is that of Rhea, who evidently utilized it in the study of the lesion of a case of fatal blastomycosis reported by Sheperd and Rhea.[17]

Of many variations tried, the technic which proved most satisfactory deviates from that regularly used in connective-tissue-staining in that the primary staining in fuchsin is somewhat decreased, that the counterstain may be not too pronounced, and the secondary staining in anilin blue mixture is greatly prolonged. Tho the usual 20 minutes' staining is sufficient for demonstrating the coarser features of the ordinary capsules, prolonged staining is required that the more delicate capsular structures may be saturated enough to withstand differentiation with 95% and absolute alcohols. When the second stain is applied for but 2 or 3 hours the results are usually less satisfactory than when this solution is used for from 12 to 24 hours. The sections most useful in the study of the forms of the blastomycetes under consideration were treated in this manner. The process stains the outer part of the cell wall a clear brilliant blue, while the protoplasm, appearing yellow to red in color, as do the erythrocytes and tissue cells, shows nothing characteristically differential. As a rule the yeast cells can easily be identified, even with low power lenses, on account of their morphology, their intensity of staining, and the fact that they usually occur within tissue cells or in exudate out of contact with confusing elements.

NOTE ON GENERAL MORPHOLOGY

Study of preparations made by this technic gives one the impression that the usual descriptions of the tissue forms of the ordinary blastomyces are more or less incomplete. In the typical organisms (to be seen in Figs. 6 and 10), one observes immediately surrounding the protoplasm and within the capsule the clear zone which has never been shown to have structure or to retain stains. In moist preparations of blastomycetes this zone seems fluid. The capsule itself, usually described as hyaline and doubly contoured, and not infrequently stated to be homogeneous, is seen to be composed typically of an inner and an outer layer, tho the latter may be absent or indistinguishable. The former, a thin flexible membrane which may be designated the capsula vera, is the true capsule, the essential covering membrane of the

[15] Jour. Med. Research, 1906, 15, p. 167.
[16] Jour. Am. Med. Assn., 1907, 49, p. 1072.
[17] Jour. Cutan. Dis., 1911, 29, p. 588.

organism, on which the outer capsula sclerotica is applied. The latter is often firm and more or less thick and may even be laminated, as in the larger central organism in Fig. 3. In ordinary gemmation the former membrane, which stains a more delicate blue than the capsula sclerotica, does not rupture but evaginates to retain the daughter cell and closes off at the time of separation. The sclerotic layer, on the other hand, often ruptures at the point of budding shortly after the process is inaugurated and remains with the mother cell. At other times, this layer in such instances being rather thin and flexible, both capsules persist about the daughter cell through its formation. This is seen in the largest budding organism in Fig. 5.

It has been interesting to study the process of capsule-formation in sections from subcutaneous lesions of an ordinary case of generalized blastomycosis. In these preparations, specially stained, are seen great numbers of ordinary, thin- and thick-walled, budding and quiescent parasites, together with numerous capsular remnants of dead organisms. These capsules or shells are often seen to persist (Fig. 6, 7, and 8), at times in much the normal form. The protoplasm, however, disintegrates and the shell may be found empty (Fig. 6) or filled with granular debris (Fig. 7). The striking feature of these capsules is the manner in which they increase in size and the extent to which this increase may be carried. It at times appears to begin as delicate knobs or buds of typical blue-staining capsular material within the shell (Fig. 6). Again, it may be less systematically applied, appearing as irregular masses (Fig. 8). In Fig. 7 the body is undergoing an irregular laminar increase. The very large body in Fig. 9 shows both the generally laminated appearance and knoblike protuberances which are themselves laminated. The flower-like body in Fig. 10 is an odd result of the process. In the two endothelial foreign-body giant cells of Fig. 11, are seen masses of the hyaline capsular material undergoing digestion.

Since after the death of a parasite the empty capsule may persist in the tissue or exudate and increase in size, sometimes to a remarkable degree, it would appear to be quite clear, that, as held by certain European writers,[13] the capsula sclerotica is not a product of the cell's vital activity but is a deposit or accumulation of a specialized material applied from without. This possibility has been mentioned by Ricketts,[18] but not emphasized. It is probable that certain of the forms described by Gilchrist and Stokes[1] are of similar nature.

[18] Quoted by Hyde, Jour. Cutan. Dis., 1901, 19, p. 44.

MORPHOLOGY OF ORGANISMS IN THE SPECIAL LESION STUDIED

The unusual lesion found in the skin of the case under considera-
tion, in which were great numbers of organisms in unusual forms,
was clearly explained by a study of sections prepared by the pro-
longed anilin-blue staining. The irregularly coarsely granular appear-
ance of the giant cells and necrotic material was seen to be due largely
to the vast number and very small size of the parasites present. The
nature and origin of these minute forms were shown by the demonstra-
tion about the majority of them of sharply drawn blue-stained capsular
membranes. These were often extremely delicate and at times defined
only by close scrutiny with the higher-power oil-immersion lenses.
Furthermore, it seems probable that not all had membranes sufficiently
distinct to resolve. While great delicacy of membrane was the rule,
this did not seem necessarily to correspond to the size of the parasite,
for some of the smaller sizes, tho not the smallest, possessed relatively
thick shells.   While the majority of these organisms were rounded,
many which presented more irregular outlines were scattered through-
out the lesion. The most of these irregular bodies which were not
explainable by pressure of surrounding structures were more or less
pear-shaped, and upon careful study were seen to be miniature bud-
ding cells.   All stages of daughter-cell-formation might be found.
Particularly noticeable was the fact that this activity was not confined
to the cells of any one stage of growth or maturation, but was seen
in individuals of all sizes and forms. This may be observed by close
scrutiny of Figs. 2 to 5 inclusive. It was not unusual to find two
cells, each but 2 or 3 microns in diameter, just about to complete the
fission process by separating, each of the daughter cells being prac-
tically identical in size and appearance and both covered by the thinest
discernible capsule. Many such dividing cellules would have been
considered two distinct organisms had it not been for the demonstra-
tion of the common cell membrane. Another small, but apparently
more mature, type of cell, measuring from but 5 to 10 microns in
diameter, might show a very thin-walled bud springing from an aper-
ture in the sclerotic layer of a cell wall as heavy as ordinarily seen on
the large sclerotic organisms. The active budding process observed
often gave the small thin-walled organisms the appearance of motility.

Granule-formation in the cytoplasm was rather constant, the
granules being made very prominent by certain stains. The smallest
forms seemed always to contain one such condensation of cytoplasm;

in them this seemed often to constitute the entire protoplasm. In
larger organisms there might be several granules present, but no sig-
nificance could be attached to them. The smaller more compact giant
cells often contained few but the smallest forms of parasites. The
individuals formed earlier seemed progressively, if slowly, to increase
in size, while the total numbers of parasites rapidly increased by the
active gemmation described. This resulted in a corresponding increase
in size of the giant cells. Ultimately these cells, filled with organisms
showing a wide range in size, underwent disintegration. Fig. 2 shows
3 stages of this process.

DISCUSSION

The early and rapid multiplication of the organisms described, by
the fission of individuals even of very small size, explains the appear-
ance of great numbers of these spore-like cellules in the lesions in
which no endosporulation was found. While budding forms of the
ordinary type were numerous, they were, as argued by LeCount and
Myers in their case, far too few to account for the vast numbers of
minute organisms found. The degree of deviation from the normal
process is more clearly appreciated when one compares the size and
numbers of the bud processes in the ordinarily active blastomycotic
lesion with those in the tissue described.

Study of the lesion in specimens stained by common methods did
not clearly eliminate the possibility of the cellules' being formed by
endosporulation. Not only were the minute budders unappreciated, but
the appearance of clusters of stained granules in old broken capsules,
giving the suggestion of sporulation, was somewhat misleading. In
the special preparations, however, tho there could be found any stage
between the smallest cellule on the one hand, and either a mature
thick-walled cell or a large thin-walled organism on the other, nowhere
was there found evidence of true endosporulation.

That the organism described may be a new variety of the blasto-
myces is held improbable. If the forms found in all the lesions were
of the peculiar type described, the possibility of its being a new species
would be worthy of consideration. There was, however, an apparent
localization of the unusual multiplication activity in the skin focus,
which is similar to the limitation to the cerebellar focus of the small
forms in LeCount and Myer's case. This feature is in accord with
the liability to morphologic variation of the blastomyces under dif-
ferent influences, particularly apparent when the organisms are arti-

ficially cultivated. While descriptions of lesser variations are numerous, Stober reports[19] that in a culture contaminated with B. subtilis he observed form variations in an otherwise ordinary strain that seemed even to have attained true sporulation. These and other facts make it probable that the unusual morphology of the organisms in the skin was due to temporary variations under the influence of unusual biologic conditions.

What the factors were which influenced this frantic multiplication cannot be asserted. The blastomycotic nature of the infection was not suspected clinically and was demonstrated only by the study of routine sections some weeks after the autopsy. But one piece of skin had been preserved and this had been removed only because the inguinal lesion did not, as did those elsewhere, resemble entirely that of pellagra. In view of the probable pellagrous condition and of the tuberculous infection of the lung, it seems likely that the blastomycotic invasion was secondary, apparently by way of the skin eroded by the pellagrous dermatitis. It is at least interesting to consider the possibility that this little-understood condition should so markedly modify the activity of the blastomyces. It is also not clear why the distribution of the blastomycotic lesions should not have been more general, in view of the probable ease of dispersion of the small form of organism found.

CONCLUSIONS

The description of the lesion considered resembles closely those of but one or possibly two lesions which have been carefully described in reports of other cases of systemic blastomycosis.

In this lesion were very numerous organisms of varying sizes, occurring diffusely and in clusters within the endothelial and giant cells and in necrotic cell debris, many of which were so small and so massed as to suggest strongly their formation by a process of endosporulation.

When specially stained, the small organisms were shown conclusively to have been formed by the ordinary budding process. This was here extremely active and, contrary to the ordinary condition, was seen in organisms of even the smallest sizes, and apparently of the most recent formation. It is not thought to indicate a variation in the species of the blastomyces.

Tho no explanation can be advanced for the frantic multiplication, it can be suggested as possibly significant that the blastomycotic infec-

[19] Arch. Int. Med., 1914, 13, p. 509.

tion was apparently secondary in skin lesions of a patient clinically a distinct pellagrin, in whom was also found pulmonary tuberculosis. The possibility that the causative factor of the pellagrous dermatitis may have also supplied the unusual stimulating influence to the blastomyces is interesting to consider.

After many attempts to stain these organisms differentially a slight adaptation of Mallory's anilin-blue connective-tissue stain was found to demonstrate them most clearly by delicate blue-stained capsular membranes. This stain, first advocated by Bowen and Wolbach, seems to be the most satisfactory process for the demonstration of the blastomycetes in the tissues. It proves useful in demonstrating details of the organisms ordinarily determined with difficulty, and in locating organisms in certain lesions where they are scarce or partially degenerated. It should be more generally utilized in the study of the lesions of this and similar infections.

For the sake of accuracy of description it seems well to emphasize the typical compound structure of the capsule of the blastomyces in the tissues, consisting as it does of an inner delicate capsula vera and usually an outer applied capsula sclerotica. The extraneous source of this outer capsular material is demonstrated by the progressive increase in size of a capsule after death of the parasite.

EXPLANATION OF PLATES

Plate 22

Fig. 1.   Field from central area of a skin lesion in the case described, showing the numbers, size, and coarsely granular appearance of the endothelial giant cells, and the practical absence of leukocytes of other types.   Destruction of the normal subcutaneous tissue is evidenced by poorly supported blood vessels among the cells of the granuloma.   Hematoxylin and eosin.   Low dry lens.

Fig. 2.   Two large giant cells, showing their coarsely granular character, which is due largely to the great numbers of very small and larger parasites, the majority too small and delicate to be clearly defined.   An old disintegrating giant cell containing several organisms of larger size is partly shown.

Fig. 3.   Field from similar lesion, with organisms apparently not so numerous and averaging considerably larger than in Fig. 2.   Several budding forms, with walls varying markedly in thickness, can be seen.

Plate 23

Figs. 4 and 5.   Other fields of the same lesion illustrating variation in the size of the organisms.   Close scrutiny demonstrates particularly well the gemmation of very small forms and the extreme delicacy of the capsular membranes of the smaller organisms.

Figs. 2 to 5 inclusive were prepared by prolonged staining with Mallory's connective-tissue stain.   Bausch and Lomb 1/16 oil immersion lens.

Figs. 6 to 11.   Nonvital deposition growth of the capsula sclerotica in dead blastomycetes.   Fig. 6 compares two normal organisms with large attenuated empty capsules which show four delicate in-growing knobs of capsular material. Fig. 7 shows the early laminar deposit upon a broken capsule filled with granular debris.   The blastomycete in Fig. 8 contains small masses of apparently unattached hyaline material among the debris.   The body shown in Fig. 9 is very large and shows both laminar and knoblike increase.   The odd flower-like body in Fig. 10 is unusual.   In Fig. 11 two endothelial giant cells enclose and are digesting such hyaline bodies.

PLATE 22

Figure 1

Figure 2

Figure 3

PLATE 23
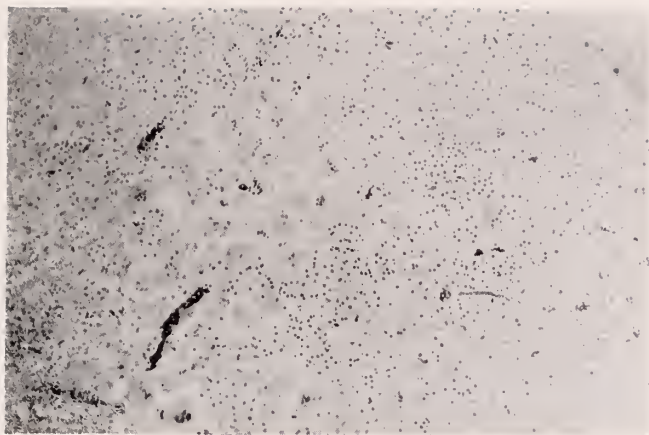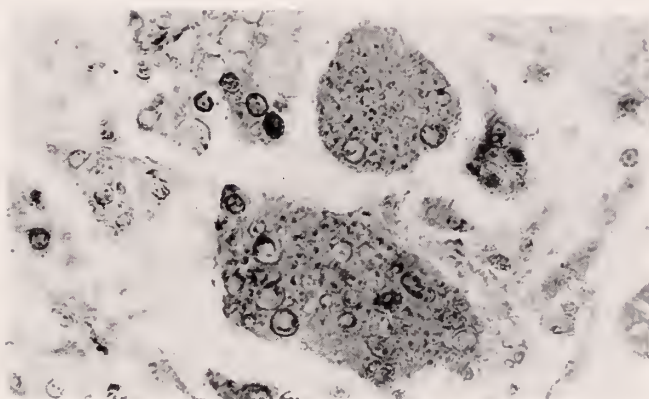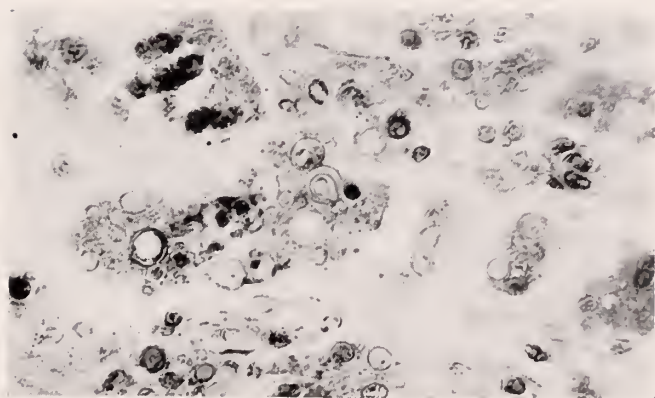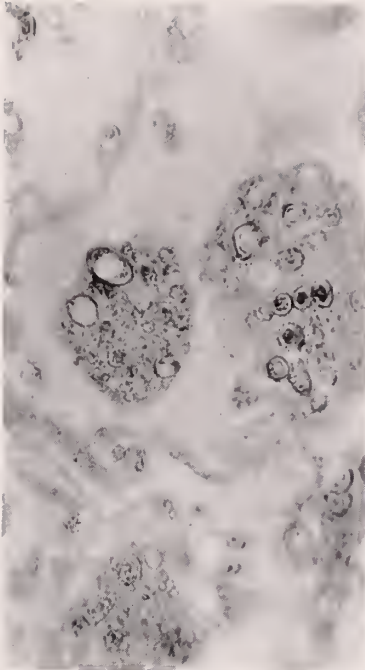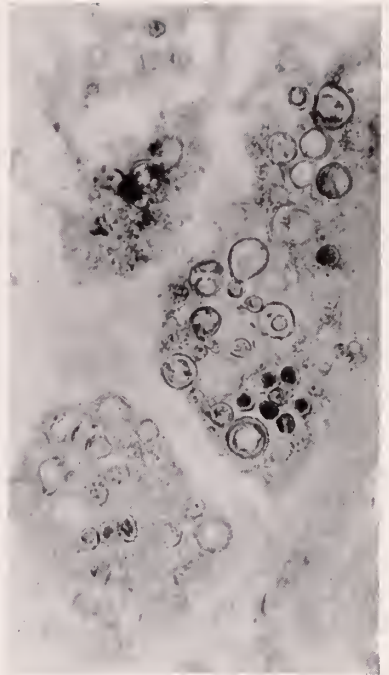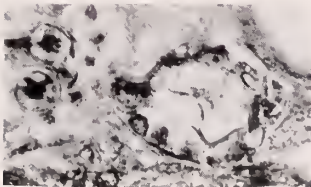
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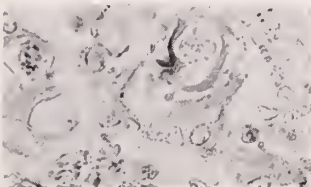
Figure 5

Figure 6

Figure 7

Figure 8
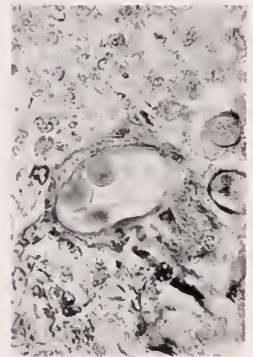
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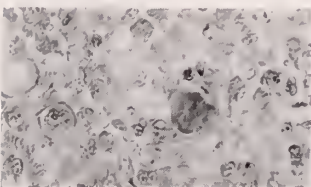
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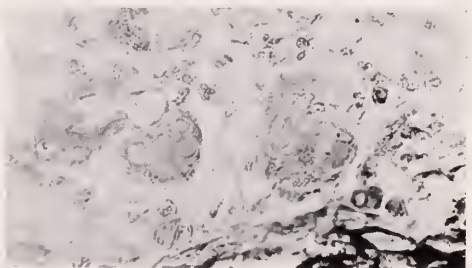
Figure 11

THE COEXISTENCE OF ANTIBODY AND ANTIGEN IN THE BODY *

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INTRODUCTION

The most striking feature of the immune reaction consists in the production of specific protective substances, known as antibodies, in response to the presence within the body of a foreign protein, the so-called antigen. These factors, indeed, have been identified, but the finer details of the mechanism of the defensive process are almost entirely obscure. The theory originally propounded, and even now generally entertained, was based on the high degree of affinity which antibody has for antigen. As rapidly as the organism could produce these protective substances, they were supposed to attack the antigen, and to neutralize or destroy it. After this process had been brought to completion, so that no free antigen remained in the body, free antibody was supposed to accumulate, and was then demonstrable as such in the blood. According to this view, the latent period which followed the introduction of antigen into an organism, and which preceded the demonstrable presence of free antibody in its blood, was occupied by the neutralization of antigen by antibody. The succeeding period, the so-called positive phase, characterized by the presence of free antibody, was taken to denote the complete neutralization of the antigen.

The validity of this theory was seriously threatened by a series of observations which seemed to indicate the coexistence of antigen and antibody within the blood over considerable periods of time.

Uhlenhuth and Weidanz,[1] and others, have noted the fact that the sera from two different rabbits, immunized against the same protein, when mixed produce a precipitate. Eisenberg[2] showed that when antigen and antibody are mixed in the test tube, the resulting precipitate does not carry down the total amount of either factor, but that both may be demonstrated in the

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[1] Technik u. Methodik des biologischen Eiweissdifferenzierungsverfahren, 1909.

[2] Centralbl. f. Bakteriol., I, O., 1903, 34, p. 259.

supernatant fluid. These two striking phenomena were interpreted as analogous; the mutual precipitation of two immune sera was regarded as evidence of the presence in both, or in one of them, of antigen in addition to antibody. Those who have believed that immunologic reactions are simply exemplifications in another field of the laws of mass action in chemistry, welcomed these facts as evidence of the correctness of their views. To Ehrlich and his followers, however, the physicochemical interpretation of the phenomena of immunization has never appeared satisfactory. In consonance with this standpoint, v. Dungern[3] has explained the observations of Linossier and Lemoine,[4] and of Eisenberg, on the basis of a fresh hypothesis. He maintains that an animal serum is not a single homogeneous antigen or protein, but a complex, or mixture, of a large number of such antigens, many of which are quite dissimilar in character. To some of these antigens it is conceivable that the antibody reaction is prompt and marked, while to others it is relatively deficient. For instance, an animal injected with a foreign serum may contain at one and the same time antigens of the latter group and antibodies to the former group. Such a situation would permit of the reactions described by these authors, whereas it by no means corresponds to their interpretation; namely, that antigen and its specific antibody may coexist in the blood. Von Dungern's theory has not been demonstrated to be true, nor has it, on the other hand, been disproved; so that the facts must still be accepted, with the reservation that their interpretation is not absolutely beyond question.

Recently this problem in the mechanism of immunity has been approached with the help of new methods by Weil.[5] In the first place, he has shown that antigen and antibody may be demonstrated at the same time in the sera of persons who have received large doses of therapeutic horse serum. In this respect, therefore, the reaction of human sera is analogous to that of the sera of the laboratory animals.

In other papers Weil[6] has studied the problem of the coexistence of antigen and antibody, not only in the blood, but also in the cells of the animal. His studies have been based entirely on the use of the methods of anaphylaxis. An interesting feature of this method consists in the fact that a positive result demonstrates the presence of antibody in a form and in amounts which are physiologically effective. The technic employed by Weil, including the use of an immune body as antigen, has been adapted to the present study, and will subsequently be described in detail. The conclusions reached by him are as follows:

After the subcutaneous administration of a large dose of a foreign serum to a guinea-pig, this serum persists demonstrably for a period of about 2 weeks, not only in the blood, but in the cells of the guinea-pig. During the latter part of this period the guinea-pig develops specific antibodies to the foreign serum, the presence of which can be demonstrated in the cells of the animal. Later on, these antibodies are demonstrable in the blood. Thus, the reaction of an organism to a foreign protein is shown to be characterized by 4 factors, all of which, except the last named, are known to coexist for a certain period of time in the body; namely, antigen in the cells, antigen in the blood, antibody in the cells, and antibody in the blood.

[3] Centralbl. f. Bakteriol., I. O., 1903, 34, p. 355.
[4] Compt. rend. Soc. de biol., 1902, 54, p. 85.
[5] Proc. Soc. Exper. Biol. and Med., 1914, 12, p. 37.
[6] Jour. Med. Research, 1914, 30, p. 299.

These factors, moreover, exert a marked influence on one another, inasmuch as they materially modify the vital reactions of the animal. A guinea-pig, the cells of which contain both antibody and antigen, responds to the addition of fresh antigen in much milder fashion than does an animal in which antibody alone is present. In the course of time, antigen disappears from the cells and from the blood, leaving only antibody, which eventually tends to disappear from the blood, but is demonstrable for an almost indefinite period in the cells. In a subsequent publication Weil[7] made use of the methods of passive sensitization to demonstrate the coexistence of antigen and antibody in the organism. As these particular studies do not touch the special problem considered in this paper, they require no further description.

Previous to the work analyzed, the study of the mechanism of immunity involved the consideration of only one factor; namely, the presence of antibodies in the serum. It seemed of importance to make a further study of the 4 factors described, and to determine, if possible, additional data as to their mode of interaction.

The problem undertaken in the present study to a certain extent involved a repetition of the work reported by Weil, with certain differences. In the first place, a different antigen was. selected. Weil had made use of the serum of rabbits immunized against horse serum. This antigen is open to the objection that the uterus of a guinea-pig highly sensitized to rabbit serum may react to horse serum. This condition was pointed out by Weil, entailing as it did certain special precautions. Nevertheless, in spite of the fact that disturbance from this factor was excluded by the experimental conditions, it seemed advisable in the present study, to select as antigen a serum containing antibodies not open to this objection. For this purpose, use was made of the serum of a rabbit highly immunized against egg albumin. There is never any question of cross sensitization as between egg albumin and rabbit serum.

Altho Weil had succeeded in demonstrating the coexistence of 3 factors in the mechanism of immunity, and had assumed the presence of the 4th factor (antibody in the serum) as already fully demonstrated by previous investigations, he did not attempt a careful analysis of the time-relationships of these factors. It is of interest to know whether the appearance of antibody in the cells antedates the appearance of the same substance in the blood. It is of considerable importance to know whether the presence of antibody in the serum does, or does not, indicate the complete disappearance of antigen from the cells. In the present study an attempt has been made to approach the solution of these questions, with, however, the distinct realization that

[7] Ztschr. f. Immunitätsf., 1914, 23, p. 1.

the methods at our disposal permit of the detection of only fairly large amounts either of antigen or of antibody.

TECHNIC AND METHODS

The technic has followed, in most respects, the methods described by Weil. In one detail of Dale's method, however, a modification has been adopted. In case of a low degree of sensitization, the uterus responds to the application of large amounts of antigen, while smaller amounts are ineffective. In testing for the presence of antibody, therefore, it is advisable to use the largest amount of antigen that will not cause a muscular contraction of the normal control uterus. In order to follow this technic, preliminary titrations were made with egg albumin and with rabbit serum, the results of which are included in this paper. It was found possible to employ in the tests amounts considerably larger than those used by Weil. As a result, passive antibody (antigen) has been shown to persist longer and active antibody to occur earlier, than was possible in previous experiments. At the same time, the period of overlapping of antigen and antibody has been extended.

Another variation of technic was attempted, with the object of demonstrating the persistence of minute traces of antigen (passive antibody) in the blood of the guinea-pig. For this purpose, Weil exsanguinated the animal and used the entire blood to sensitize a 2nd guinea-pig passively. Obviously, the success of this experiment requires the persistence of considerable amounts of antigen. The attempt was therefore made to demonstrate the antigen by testing the blood of the guinea-pig against the uterus of a guinea-pig sensitized to the antigen in question. Thus, if it is desirable to test for the persistence of antigen in a guinea-pig which has received an injection of the serum of a rabbit immunized against egg albumin, the blood of that guinea-pig is added, in proper amount, to the uterine preparation of a guinea-pig sensitized towards rabbit serum (Figs. 9 and 10). A contractile response reveals the persistence of the rabbit component in the blood of the 1st guinea-pig. The method, which had been previously used, is sensitive and accurate.

Preparation of Immune Serum.—Rabbits were immunized against egg albumin by daily, or almost daily, intravenous inoculation of gradually increasing doses of the antigen. A typical report is given in Table 1. On January 25, a small quantity of blood was taken from the ear vein, and the serum injected as shown in Table 2.

TABLE 1

IMMUNIZATION OF RABBIT 564

Date of Inoculation	Dose in c.c. of Egg Albumin
Jan. 9	0.2
Jan. 11	0.4
Jan. 12	0.4 and again after ½ hour
	0.4
Jan. 13	0.4 and again after ½ hour
	0.6
Jan. 15	0.6
Jan. 18	0.6 and again after ½ hour
	0.4

TABLE 2

Results of Sensitization of Guinea-Pigs to Egg Albumin

Guinea-pig	Jan. 25 (Rabbit serum 564 injected intraperitoneally)	Jan. 27 (Egg albumin injected intravenously)	Result
518	0.25 c.c.	0.5 c.c., 50% egg albumin (diluted with salt solution)	Died immediately
519	0.1 c.c.	0.5 c.c., 50% egg albumin (diluted with salt solution)	Died immediately

Table 2 shows that the sensitizing dose (or titer) of this serum is 0.1 c.c. or less. A serum of lower titer was never used. The guinea-pigs used in the experiments to be outlined, usually received 3 c.c. of the immune serum intraperitoneally, in other words, 30 sensitizing doses, or more.

Methods of Determining the Presence of Antigen and Antibody. — The essential feature in the experiments is the use of an immune serum as antigen. Thus, guinea-pigs were prepared by the injection of 3 c.c. of rabbit serum immunized against egg albumin. The animals were killed by exsanguination at intervals varying between 9 and 24 days after injection, and tests were made at once for intracellular antigen and intracellular antibody. The uteri were suspended in a Dale apparatus. Antigen was demonstrated by uterine contraction following the addition of egg albumin to the Locke's fluid bathing the uterus. Antibody was demonstrated by uterine contraction following the addition of rabbit serum. The reaction of the uterus to egg albumin indicates the presence of rabbit serum in the uterine cells, for the guinea-pig cells contain antibodies for egg albumin only by virtue of the absorption of serum immunized against egg albumin. The reaction of the uterus to rabbit serum reveals the production by the guinea-pig of antibodies to the injected rabbit serum. Briefly, then, the response to egg albumin indicates the presence of antigen, and the response to rabbit serum, of antibody.

The demonstration of circulating antigen and circulating antibody involves the use of similar methods. The serum obtained by exsanguination of the guinea-pigs inoculated with rabbit serum versus egg albumin, is injected into normal virgin pigs intraperitoneally. After 3 or 4 days these pigs are killed and the uteri are tested for antigen and antibody, as outlined. Certain differences involved in the demonstration of circulating antigen and antibody as compared with the demonstration of the cellular elements, will be considered more fully with the description of the individual experiments.

Before these experiments could be begun, certain preliminary tests were necessary. The uterus of a normal guinea-pig will contract on the addition of any serum, provided that a sufficient amount be employed. It was therefore necessary to determine the largest amounts of egg albumin and rabbit serum that could be used without causing a contraction in a normal uterus. It was found that as much as 3 c.c. (Fig. 1) and 5 c.c. (Fig. 2) of egg albumin could be added without causing contraction in a normal uterus. Amounts of rabbit serum up to 0.5 c.c. (Fig. 1) consistently failed to cause contraction. As a rule, 0.7 c.c. and 1 c.c. also failed to elicit a response, but because of an occasional contraction when the latter quantity was used, 0.5 c.c. of rabbit serum was decided upon as the largest quantity to be employed in testing for the presence of antibody.

THE PRESENCE AND PERSISTENCE OF ANTIGEN IN THE CELL

The following is the method by which cellular antigen is demonstrated. Guinea-pigs are injected, intraperitoneally, with 3 c.c. of rabbit serum immunized against egg albumin. At varying intervals of time after the injection, the animals are exsanguinated and the uteri suspended in a Dale apparatus. Contraction following the addition of egg albumin to the fluid surrounding the uterus, indicates the presence of antibodies for egg albumin in the uterine cells; the presence, in other words, of the rabbit serum component—antigen (Fig 3).

TABLE 3

Cellular Antigen

Guinea-pig	Days After Injection	Reaction Present + or Absent 0	Amount in c.c. of Egg Albumin Used to Elicit Reaction
104	9	+	0.1
120	9	+	0.3
121	11	+	0.4
123	11	+	1.8
714	11	+	0.8
685	12	+	0.75
127	14	+	1.5
525	17	+	1.2
719	18	0	2.0
529	21	0	1.8
526	24	0	1.5
...	..	..	...
103	12	0	0.2
122	13	0	0.3

These experiments cover a period from the 9th to the 24th day after the injection of rabbit immune serum. Antigen has been demonstrated from the 9th (Figs. 3 and 4) to the 17th day (Fig. 5) ; after the 17th day the antigen disappears from the cell, as indicated by the fact that reactions with large doses of antigen could not be obtained on the 18th, 21st, and 24th days. Altho not tested previous to the 9th day, it is evident that antigen must be present from the 2nd day after injection, as passive sensitization occurs within 24 hours. It is important to note that the demonstration of antigen requires increasingly large amounts of egg albumin the longer the interval after the sensitizing injection. For example Table 3 (see also Figs. 3, 4, and 5) shows the following: Uterus of Guinea-pig 104, 9 days after injection, reacted to 0.1 c.c. egg albumin. Uterus of Guinea-pig 103, 12 days after injection, failed to react to 0.2 c.c. egg albumin. But uterus of Guinea-pig 685, also 12 days after injection, responded to 0.75 c.c. egg

albumin. Further, uterus of Guinea-pig 525, 17 days after injection, responded to 1.2 c.c. egg albumin, whereas uterus of Guinea-pig 719, 18 days after injection, failed to respond to 2 c.c. egg albumin. After the 17th day, then, antigen apparently disappears from the cell, or, at any rate, can no longer be demonstrated in the cell by methods which were effectual before that time.

Table 3 presents the results of a series of tests in detailed form.

THE OCCURRENCE AND PERSISTENCE OF ANTIBODY IN THE CELL

The preparation of the guinea-pigs for the demonstration of cellular antibody is identical with that described in the previous section, as the same uteri which were tested for cellular antigen were used to demonstrate the presence of antibody. Rabbit serum was added to the Locke's fluid, and a uterine contraction indicated the presence in the cell of antibodies to rabbit serum.

The experiments cover a period from the 9th to the 24th day after injection, and antibody has been demonstrated during this entire time (Table 4 and Figs. 3 and 4). The antibody was presumably present even before the 9th day, but as no experiments were performed before that time, the earliest demonstrable appearance of cellular antibody is not definitely known. Cellular antibody persists after the 24th day, probably indefinitely. The experiments of Anderson, referred to in the first part of this paper, indicate the persistence of antibodies during the entire life of the guinea-pig. Table 4 presents in detail the results of these tests.

TABLE 4

CELLULAR ANTIBODY

Guinea-pig	Days After Injection	Reaction Present +	Amount in c.c. of Rabbit Serum Used to Elicit Reaction
104	9	+	0.1
120	9	+	0.5
121	11	+	0.3
123	11	+	0.3
685	12	+	0.3
122	13	+	0.3
127	14	+	0.5
525	17	+	0.4
719	18	+	0.3
529	21	+	0.5
526	24	+	0.3

THE PERSISTENCE OF CIRCULATING OR FREE ANTIGEN

The method (another method will be described in the latter part of this paper) of demonstrating circulating antigen is as follows: Guinea-pigs are prepared as described; that is, 3 c.c. of rabbit serum immunized against egg albumin are injected intraperitoneally. After varying intervals the pigs are exsanguinated, and the serum obtained is injected intraperitoneally into another guinea-pig. The latter pig is killed after 3 or 4 days, and the uteri are tested for the presence of antigen (by the reaction to egg albumin) and for the presence of antibody (by the reaction to rabbit serum). As an example of the method, one such experiment will be described in detail.

Guinea-pig 104 received 3 c.c. of rabbit serum (Rabbit 291, highly immunized against egg albumin). After an interval of 9 days, this guinea-pig was killed by exsanguination, and the uteri were tested for cellular antigen and antibody (Fig. 3), both of which were demonstrated. The 2 c.c. of serum, obtained by exsanguination, were injected intraperitoneally into Guinea-pig 65. Three days later Guinea-pig 65 was killed and the uteri were tested for antigen and antibody. Fig. 6 shows a marked reaction to egg albumin, indicating the presence of a rabbit serum component (antigen) in the blood of Guinea-pig 104. No reaction was obtained on the addition of rabbit serum, a result indicating that on the 9th day antibody had not appeared in the circulating blood in an amount sufficient to confer passive sensitization.

Briefly, then, Guinea-pig 104 demonstrates that on the 9th day antigen persisted in both cells and circulation, and that antibody had been produced and was demonstrable in the cells, but had not as yet appeared in the blood.

TABLE 5

Free or Circulating Antigen

Guinea-pig	Days After Injection	Reaction Present + or Absent 0	Amount in c.c. of Egg Albumin Used to Elicit Reaction
104	9	+	0.8
120	9	+	0.4
121*	11	+	2.0
685	12	+	3.0
525	17	+	1.8
719	18	0	2.5
529	21	0	1.5

* Presence of free antigen indicated also by the reaction of 0.5 c.c. of the serum of Guinea-pig 121 on uterine preparation of Guinea-pig 1260, which was actively sensitized against rabbit serum.

This method of demonstrating circulating antigen has very definite limitations. It is not satisfactory for showing small amounts, as enough must be present to sensitize a guinea-pig passively. The results depend to a considerable degree on the quantity of blood recov-

ered on exsanguination; if the yield is small, the demonstration of the immune body may fail. These factors explain the irregularity of the results.

The antigen is of course present in relatively large amounts in the blood during the 1st week after sensitization. Its persistence can be demonstrated for 17 days (see Figs. 7 and 8). Tests made on the 18th and 21st days after injection fail, however, to show the presence of circulating antigen. Table 5 presents details of these experiments.

THE OCCURRENCE AND PERSISTENCE OF CIRCULATING ANTIBODY

The method is identical with that just described for the demonstration of circulating antigen; rabbit serum instead of egg albumin, is used to elicit the reaction. The same limitations obtain in the demonstration of circulating antibody as were just mentioned with reference to free antigen, since the results depend in considerable degree on the amount of blood recovered by exsanguination.

Antibody does not appear in the circulation until the 14th day (Table 6), but is constantly present after that time (Fig. 8). The experiments cover a period of only 21 days; antibody is known to persist in the blood for a longer period, but the extreme limit of its occurrence has not been tested by this method.

TABLE 6

Free or Circulating Antibody

Guinea-pig	Days After Injection	Reaction Present + or Absent 0	Amount in c.c. of Rabbit Serum Used to Elicit Reaction
104	9	0	0.5
120	9	0	0.4
121	11	0	0.5
685	12	0	0.5
127	14	+	0.5
525	17	+	0.5
719	18	+	0.55
529	21	+	0.5

INTERRELATIONSHIP OF FOUR FACTORS

The time relationship of the 4 factors may be best appreciated by roughly grouping the appearance and disappearance of each of them into 4 periods of 1 week each (Table 7 and Chart 1). In the 1st week antigen is present, both in the blood and in the cells; in the 2nd week antibody appears in the cells, and the cellular and circulating antigen persist; in the 3rd week, antibody appears in the blood and antigen disappears from both cells and blood; antibody persists in the cell; in the 4th week, antibody is present both in the cells and in the blood, while antigen is no longer demonstrable in either.

For a period of more than a week antigen and antibody coexist in the cells.   For several days antigen and antibody coexist in the circulation. During a short period of a few days all 4 factors are demonstrable together. These relations are more convincingly demonstrated by data illustrating the occurrence of all 4 factors in individual animals. Thus, Guinea-pig 525 received 3 c.c. of rabbit serum versus egg albumin. Seventeen days later the uteri of this animal showed the presence of both antigen and antibody (Fig. 5). The serum recovered by exsanguination of Guinea-pig 525 was injected into Guinea-pig 590. Four days later this animal was killed; Figure 8 shows the presence of antigen and antibody in its uterine cells. This indicates that antigen and antibody were present in the serum of Guinea-pig 525, which was used to sensitize Guinea-pig 590. Thus, the coexistence of all 4 factors was demonstrated in one animal.

Table 7 presents these data in detailed form. Chart 1 gives a graphic illustration of the facts.

Chart 1.   The persistence of cellular and circulating antigen and the occurrence of cellular and circulating antibody.   Cellular antigen may be assumed to be present before the 9th day, tho no test was made for it by this method.   No test was made for cellular antibody before the 9th or after the 24th day, and none for free or circulating antibody after the 21st day.   Between the 14th and 17th days, the lines indicating the presence of cellular antigen and antibody and circulating antigen and antibody overlap; in other words, for a short period of time (14 to 18 days), these 4 factors in immunity coexist.

TABLE   7

APPEARANCE, PERSISTENCE, AND DISAPPEARANCE OF ANTIGEN AND ANTIBODY

Guinea-pig	Days After Injection	Cellular Antigen	Cellular Antibody	Circulating Antigen	Circulating Antibody
104	9	+	+	+	0
120	9	+	+	+	0
121	11	+	+	+	0
685	12	+	+	+	0
127	14	+	+	?	+
525	17	+	+	+	+
719	18	0	+	0	+
529	21	0	+	0	+
526	24	0	+		

DEMONSTRATION OF FOREIGN ANTIGEN IN THE BLOOD

Altho not absolutely essential to the present study, two other types of experiments will be outlined.

The first is a method for demonstrating circulating antigen in small amounts of serum. It will be remembered that the demonstration of circulating antigen was rendered difficult and uncertain because a large amount of serum was required whereas the amounts that actually were recovered on exsanguination of the sensitized guinea-pig were variable. The method to be described demands only 0.5 c.c. serum. The rationale of the procedure is as follows: The blood of a guinea-pig which contains a rabbit serum component will elicit a contraction when added to the uterine preparation of an animal sensitized to rabbit serum; that is, a uterus the cells of which possess antibodies to rabbit serum.

The detailed description of one such experiment will demonstrate the mechanism of the method. A preliminary tracing is necessary to show that the uterus to be used in the experiment proper does actually respond actively to rabbit serum. Guinea-pig 1260 was injected $2\frac{1}{2}$ months previously with 0.5 c.c. rabbit serum. The animal was killed and one horn of the uterus was placed in a Dale apparatus (Fig. 9). To this uterus 0.01 c.c. rabbit serum was added. The reaction indicates the presence of antibodies to rabbit serum in the uterus of Guinea-pig 1260. The other horn of this animal's uterus was used for the test.

The preparation of the animal which was to be tested for circulating antigen, was as follows: Guinea-pig 121 received 3 c.c. of rabbit serum vs. egg albumin, intraperitoneally. After an interval of 11 days the animal was exsanguinated and 1 c.c. reserved for this experiment. The second horn of the uterus of Guinea-pig 1260 was placed in a Dale apparatus. To this 0.5 c.c. of serum (Guinea-pig 121) was added (Fig. 10). The reaction indicates the presence of a rabbit serum component (antigen) in the serum of Guinea-pig 121.

COEXISTENCE OF FACTORS IN DESENSITIZATION

The second type of experiment which was mentioned as not being essential to the present study, but which is nevertheless of importance, involves the demonstration of the coexistence of cellular antigen and cellular antibody in partial desensitization.

The rationale of this procedure is as follows: A guinea-pig is sensitized to rabbit serum, and after an interval of several weeks, the desensitizing injection is given. However, the antigen used for this 2nd injection is itself an immune body. Depending on the conception of the interaction of antigen and antibody, two possibilities present themselves. The substance which is present in excess will completely neutralize the other, and either antigen alone or antibody alone will be demonstrated. Or, the two factors—antigen and antibody—will exist together. The latter has proved to be the correct view.

A detailed description of an experiment will illustrate the method.

Guinea-pig 363 was injected with 0.5 c.c. rabbit serum intraperitoneally. After an interval of 10 weeks the guinea-pig received the desensitizing injection; namely, 1 c.c. rabbit serum (Rabbit 291 highly immunized to egg albumin) intraperitoneally (Fig. 11). Two days later the guinea-pig was killed and the uteri were suspended in a Dale apparatus. The uterus contracted on the addition of egg albumin and of rabbit serum, thus indicating the presence in the cells both of rabbit serum (the antigen) and of antibodies to rabbit serum (the antibody) simultaneously. This result agrees with Weil's findings.

THEORETICAL CONSIDERATIONS

The entire period of reaction covered by these studies is 4 weeks. At the end of that time demonstrable antigen has disappeared from the cells and the blood. Antibody at the end of this period is present in large amount, both in cells and blood. But the complete evolution of the phenomenon is even now not perfected. During the succeeding periods there would be demonstrable a gradual fall in the antibody content in the blood, until a level is reached at which available methods would fail to reveal it. Coincidently, there is a drop in the cellular antibody, which, however, persists, according to Rosenau and Anderson, in sufficient amount to mediate anaphylactic shock for a period of 3 years—practically during the rest of the animal's life.

Hence, the mechanism of reaction to the injection of a foreign protein is a very complex phenomenon. In the present study the primary injection was rather large, namely, 3 c.c. There is every reason to believe that with smaller doses an essentially similar mechanism comes into play, altho it is readily conceivable that the time relationship of the various factors would present material differences.

The application of these data to infectious disease is necessarily imperfect because of the inadequacy of our knowledge with respect to the 4 factors concerned. There are certain facts, however, which indicate a similar mechanism. For instance, in typhoid fever, it is well

known that in a very high percentage of the cases blood cultures are positive early in the disease, while they tend to become negative in increasing degree as the disease progresses. The agglutination test for typhoid bacilli, however, pursues an inverse course; it is negative, as a rule, in the early stages, and becomes positive in a large proportion of cases as the disease progresses. The external reaction, as shown by Gay[8] and Austrian,[9] takes a course similar to that of the agglutination test in the blood. If, now, we consider the bacillus as the antigen, the agglutination test as evidence of circulating antibody, and the skin reaction as evidence of cellular antibody, it is evident that in general the mechanism presents striking analogies with those experimentally demonstrated. As an actual fact, the same patient may simultaneously present a positive blood culture and a positive agglutination test. It is freely admitted that this comparison is to some degree hypothetic as regards the identification of the several factors, but it presents suggestive features which may be of value in the understanding of infectious diseases. No less interesting is the observation recently made in a study of typhus fever, that during the early period of convalescence the blood may contain the typhus organism at the same time that it contains agglutinins thereto (Plotz, Olitzky, and Baehr[10]). In the same way Weil has shown that the injection of a large amount of therapeutic horse serum in a case of meningitis was followed by a period during which both the horse serum and antibodies thereto were demonstrable in the blood. At the present time the data bearing on this subject are extremely meager, but it seems likely that they will eventually accumulate in sufficient amount to throw new light on the reaction to infections.

CONCLUSIONS

Antigen both in the cells and in the blood, and antibody, likewise, in the cells and in the blood, may be demonstrated during a period of 3 weeks succeeding the injection of a foreign serum into a guinea-pig.

Antigen in the cells has been demonstrated for a period of 17 days after injection; antigen in the blood also for 17 days. After this time these factors apparently disappear.

Antibody is demonstrable in the cells from the 9th day onward. Antibody is demonstrable in the blood after the 14th day.

[8] Publ. in Path. Univ. of California, 1913, 2, p. 127.

[9] Bull. Johns Hopkins Hosp., 1912, 23, p. 1.

[10] Jour. Infect. Dis., 1915, 17, p. 52.

The interrelations of these 4 factors are probably very complicated. For a period of several days all may coexist in the body.

The observation is confirmed that after partial desensitization both antigen and antibody are demonstrable in the cell.

EXPLANATION OF PLATES 24 AND 25
Plate 24

Fig. 1.   A control experiment showing that 3 c.c. of egg albumin and 0.5 c.c. of rabbit serum produce no contraction when added to Locke's fluid surrounding a normal uterus.

Fig. 2.   A control experiment showing that 5 c.c. of egg albumin do not produce a contraction in a normal uterus.   The reaction to ergamine indicates the excellent contractility of this uterine muscle.

Fig. 3.   The coexistence of antigen and antibody in the cell 9 days after injection.   Guinea-pig 104 received, intraperitoneally, 2 c.c. of rabbit serum (Rabbit 291 highly immunized against egg albumin).   After 9 days the guineapig was killed.   Uterus responded to the addition of 0.1 c.c. egg albumin (thus indicating the presence of antigen) and to 0.1 c.c. rabbit serum (thus indicating the presence of antibody).

Fig. 4.   The presence of antigen and antibody in the cell 11 days after injection.   On October 17 Guinea-pig 121 received, intraperitoneally, 3 c.c. rabbit serum (Rabbit 291 highly immunized against egg albumin).   After 11 days the animal was exsanguinated.   The uteri were removed and tracings made.   The response to 0.4 c.c. egg albumin indicates the presence of rabbit serum in the uterine cells (antigen) and the response to 0.3 c.c. rabbit serum, of antibodies to rabbit serum (antibody).   The minimal contractions after the subsequent addition of 0.7 c.c. egg albumin and 0.6 c.c. rabbit serum indicate complete desensitization resulting from the first doses.

Fig. 5.   The coexistence of antigen and antibody in the cell, 17 days after injection.   On January 27, Guinea-pig 525 received, intraperitoneally, 3 c.c. of rabbit serum (Rabbit 564 highly immunized against egg albumin) ; 17 days later the animal was exsanguinated.   The uteri were placed in a Dale apparatus and tested for antigen and antibody.   The reaction to 1.8 c.c. egg albumin indicates the persistence of rabbit serum (antigen), and the response to 0.4 c.c. rabbit serum, demonstrates antibodies to rabbit serum (antibody).   The latter feature being of the routine type in actively sensitized guinea-pigs is not reproduced.

Fig. 6.   The presence of antigen in the circulation, 9 days after injection. Guinea-pig 65 received 2 c.c. of serum recovered by exsanguination of Guineapig 104.   (Nine days previously Guinea-pig 104 had received 2 c.c. of rabbit serum versus egg albumin.)   After an interval of 3 days Guinea-pig 65 was killed and the uteri suspended in a Dale apparatus.   The reaction to 0.8 c.c. egg albumin indicates the presence of a rabbit serum component (antigen), which represents the passive absorption of the injected serum of Guinea-pig 104.

Fig. 7.   The persistence of antigen in the blood 11 days after injection. Guinea-pig 137 received 2.25 c.c. of serum (Guinea-pig 121) intraperitoneally. (Eleven days previously Guinea-pig 121 had received 3 c.c. of rabbit serum versus egg albumin.)   After 2 days Guinea-pig 137 was killed, and tracings made.   The response to 2 c.c. egg albumin indicates the presence of a rabbit

serum component, which represents the passive absorption of the injected serum (Guinea-pig 121). Also, the absence of a response on the addition of 0.5 c.c. rabbit serum (shown in the tracing) indicates that circulating antibody had not appeared on the 11th day.

PLATE 25

FIG. 8. The presence of antigen and antibody in the blood 17 days after injection. Guinea-pig 590 received 2.25 c.c. of serum of Guinea-pig 525. (Seventeen days previously Guinea-pig 525 had received 3 c.c. rabbit serum versus egg albumin, Fig. 5.) Four days later Guinea-pig 590 was killed and tracings made. The reaction to 1.8 c.c. egg albumin indicates the presence of rabbit serum (antigen) and the response to 0.5 c.c. rabbit serum demonstrates antibodies to rabbit serum (antibody). Thus Figs. 5 and 8 show the coexistence of all 4 factors in one animal. Fig. 5 demonstrates the simultaneous presence of cellular antigen and cellular antibody and Fig. 8 of circulating antigen and circulating antibody.

FIG. 9. Preliminary experiment showing the presence of antibodies against rabbit serum in the uterus of Guinea-pig 1260. Guinea-pig 1260 received 0.5 c.c. rabbit serum, intraperitoneally, on August 13. Two and one-half months later the animal was killed and the horns of the uterus were suspended in the Dale apparatus. The reaction to 0.01 c.c. of rabbit serum indicates the presence of rabbit antibodies in high concentration.

FIG. 10. The presence of antigen in the blood. The addition of 0.5 c.c. serum of Guinea-pig 121 to a uterine preparation (Guinea-pig 1260) causes a contraction. This indicates a rabbit serum component (antigen) in the serum of Guinea-pig 121, as rabbit antibodies had been demonstrated in the opposite horn of Guinea-pig 1260 (Fig. 9).

FIG. 11. The demonstration of the coexistence of antigen and antibody in the cell by the method of desensitization. Guinea-pig 363 received 0.5 c.c. rabbit serum, intraperitoneally, on August 13. Ten weeks later 1 c.c. of rabbit serum (Rabbit 291 highly immunized against egg albumin) was injected intraperitoneally. After 2 days Guinea-pig 363 was killed and tracings made. Response to egg albumin indicates the presence of rabbit serum (antigen) and the response to rabbit serum indicates the persistence of antibodies to rabbit serum (antibody).
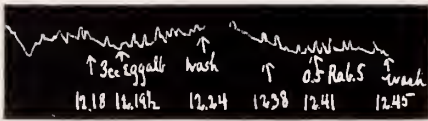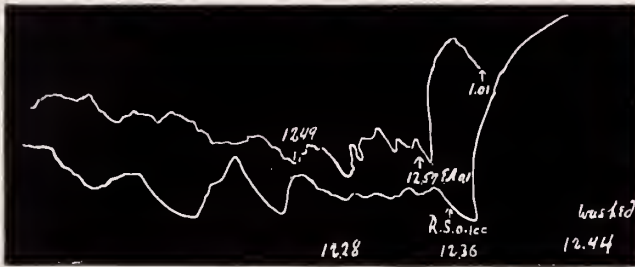
PLATE 24
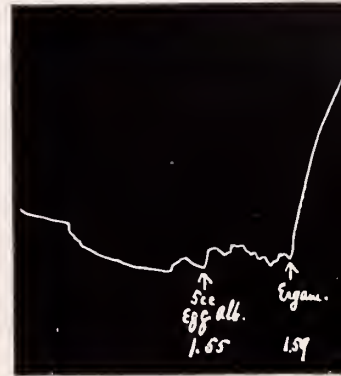
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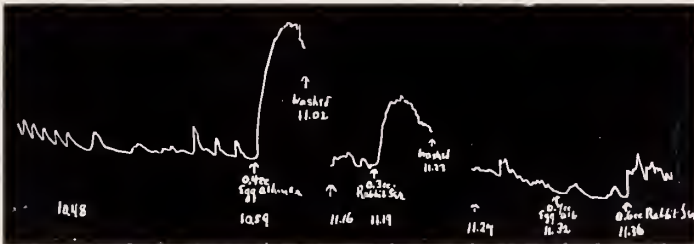
Figure 2

Figure 3

Figure 4
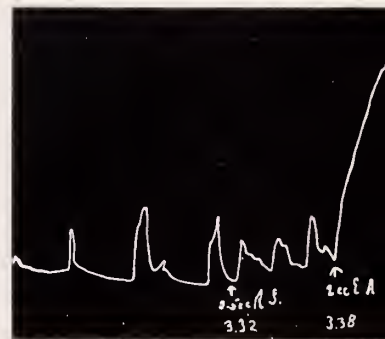
Figure 5

Figure 6

Figure 7

PLATE 25
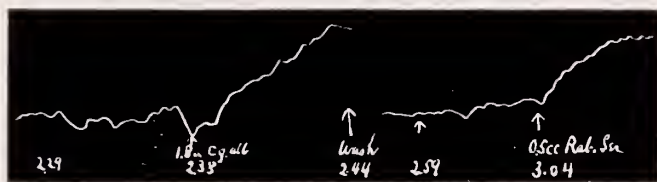
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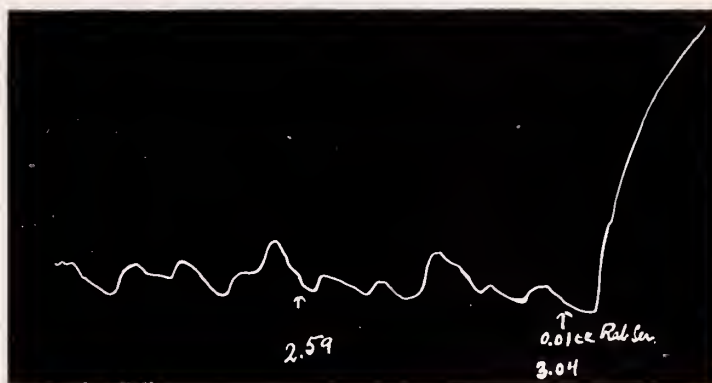
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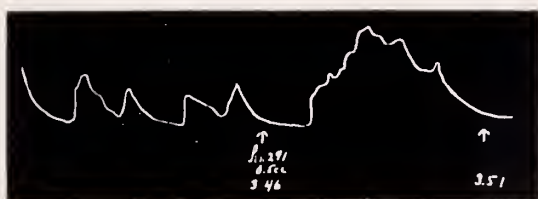
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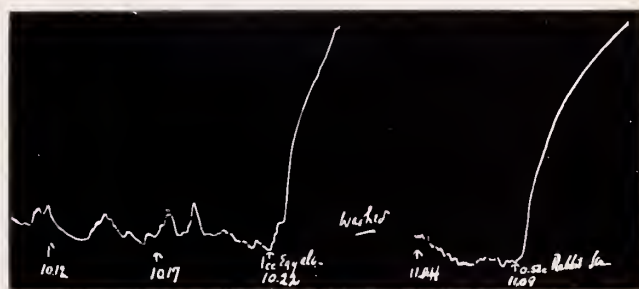
Figure 11

AN IMPROVED BRILLIANT-GREEN CULTURE MEDIUM FOR THE ISOLATION OF TYPHOID BACILLI FROM STOOLS *

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The great variety of methods that have been recommended for the isolation of typhoid bacilli from stools is strong evidence that none of these methods is thoroughly satisfactory.

Three general types of media have been devised for this purpose:

1. Solid media on which there is a sharp differentiation between lactose-fermenting and non-lactose-fermenting colonies, but no inhibition of the growth of organisms accompanying the typhoid bacilli. The Conradi-Drigalski and Endo plates have displaced all others in this group. Harris and Teague[1] have recently devised a methylene-blue eosin plate which also belongs in this group and which has certain advantages over the two plates just mentioned.

2. Solid media which inhibit the growth of many strains of B. coli and of other fecal bacteria to a much greater extent than they inhibit B. typhosus. Many varieties of malachite-green agar and Conradi's brilliant-green agar belong here. These media were popular for a while but gradually fell into disuse for reasons which will be discussed later in this paper.

3. Fluid enriching media which allow the typhoid bacilli to multiply more rapidly than the accompanying fecal bacteria. Caffein broth, bile, and Browning's brilliant-green peptone solution represent attempts in this direction, but they must be regarded as failures.

Brilliant green was found by Conradi to have a marked inhibitive action on B. coli at dilutions which would allow typhoid bacilli to grow, and its action in this respect was more selective than that of any other dye tried by him. Krumwiede and Pratt state that a number of the green dyes show this differential action, but that at the appropriate dilution no one of them is more differential than another. In our hands solid green dissolved in nutrient agar was as selective as brilliant green, but the malachite greens were inferior. It may be accepted, therefore, that as yet no stain has been found to have a greater selective action toward the typhoid bacilli than has brilliant green.

But while the majority of strains of B. coli are more readily inhibited by brilliant green than is the typhoid bacillus, yet the reverse is not infrequently the case and for the aerogenes group this seems to be the rule rather than the exception.

Altho brilliant green is far more selective in this sense than are the related violet dyes, nevertheless certain strains of B. coli that are resistant to brilliant green are inhibited by some of the latter stains. Furthermore, these violet dyes differ among themselves, some inhibiting one strain of B. coli, some

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[1] Jour. Infect. Dis., 1916, 18, p. 596.

another, as is illustrated in Table 1. Pure cultures of strains of B. coli known to be resistant to brilliant green were suspended in salt solution, one loop of which was inoculated on each of the plates. The largest amount of stain which, dissolved in nutrient agar of reaction + 1, allowed B. typhosus to grow, was employed in each instance. The readings were made after 48 hours' incubation; zero thus indicates complete inhibition of growth.

It is seen from the table that the several strains behave differently in the presence of different dyes. These are of course selected strains; many strains that are resistant to brilliant green are resistant to all of these violet dyes as well.

It seemed likely, therefore, that by adding certain of these stains in proper amounts, to brilliant-green agar, there would be obtained a much more complete inhibition of the fecal bacteria. Many such mixtures have been prepared by us, but none of them has yielded as good inhibition when inoculated with feces as can be secured with brilliant-green agar alone.

Brilliant green dissolved in ordinary nutrient agar of reaction + 1 to the amount of 1/3000% makes an excellent medium for the isolation of typhoid bacilli from the majority of stools. It is superior to Conradi's brilliant-green medium, since the increased acidity of the latter and the picric acid contained diminish rather than heighten the selective action of the brilliant green. The weakness of brilliant-green agar lies in the fact that when a specimen of feces containing colon bacilli resistant to brilliant green is encountered, numerous colonies develop on the plate, and, as there is but little difference between the appearance of some of these colonies of B. coli and that of colonies of B. typhosus, the result is less satisfactory than on the Endo plate. Another point of importance is that the margin of safety with regard to the amount of brilliant green to be used is not wide: if too much is employed the typhoid colonies develop slowly—sometimes only after 48 hours' incubation—and are reduced in number; if too little, the colon bacilli are not adequately inhibited. Furthermore, different lots of agar containing brilliant green in exactly the same concentrations may yield somewhat different results. A cloudiness in the agar renders a certain amount of the brilliant green inactive and so it is conceivable that small particles distributed throughout the agar in quantities insufficient to produce a noticeable cloudiness might yet absorb an appreciable amount of the stain. In spite of these dangers brilliant-green agar yields excellent results with most stools, allowing the inoculation of comparatively large amounts of the feces and showing often only typhoid colonies or typhoid colonies and a few opaque colonies readily distinguishable from them. But since one cannot know beforehand whether or not a given stool is going to be the exception and yield too many colonies of B. coli on the brilliant-green plate, one hesi-

tates to use this plate as a routine procedure. We have had no experience with the various malachite-green media that have been recommended, but we judge that they are inferior to the brilliant-green agar and that the same dangers attend their use. This would explain their gradual lapse from popularity to disfavor as their shortcomings were appreciated. There is no doubt that brilliant-green agar employed together with the Endo plate will yield much better results than the Endo plate alone.

TABLE 1

The Variation Among Dyes in their Inhibition of Different Strains of B. Coli and B. Typhosus

Culture	Brilliant Green .0005%	Victoria 4 R .025%	Ethylviolet .025%	Methylviolet 6 B .025%	Dahlia .025%
B. typhosus	+	+	+	+	+
F. endo 2....	+	+	0	±	+
Aerog. J. B.	+	+	±	0	0
T. d.	+	+	0	+	+
Aerog. 4.....	+	0	+	+	+

We have devised a medium which has practically the same selective action for the typhoid bacillus as brilliant-green agar and which at the same time differentiates between the lactose-fermenting and the non-lactose-fermenting colonies. The following experiment illustrates the principles on which the method rests:

Ordinary nutrient agar containing meat infusion, 1% peptone, 0.5% sodium chlorid, and 1.5% agar was prepared in the autoclave, cleared with egg, and put into flasks. It was then sterilized in the autoclave and stored for use. After sterilization it titrated +0.9. The agar was melted and 1% of saccharose and 1% of lactose were added to it. A 1% solution of brilliant green in 50% alcohol and a 3% solution of yellow eosin in distilled water were prepared. Further dilutions of brilliant green in distilled water were made so that 1 c.c. of each added to 50 c.c. of agar yielded the final dilution of brilliant green indicated in the table. The eosin solution was added to some of the agar in the proportion of 1 c.c. to 50 c.c. and the brilliant-green solutions were added to this eosin agar to yield the final concentrations of brilliant green indicated in the table; namely, 0.003%, 0.005%, and 0.01%. Plates were poured from each dilution of the stain in agar as soon as it was prepared.

To another portion of this same lot of nutrient agar a thick suspension of feces, previously sterilized in the autoclave, was added until the whole mass of agar looked somewhat cloudy. Dilutions of bril-

liant green, and of eosin and brilliant green, were prepared in this agar plus feces in exactly the same manner as in clear agar, and plates were poured as before. Before inoculation the excess of moisture was removed by placing the uncovered plates face down in the incubator for about 20 minutes.

Portions of several fresh normal stools, rubbed up in salt solution, were filtered through a thin layer of absorbent cotton, and the mixture was then diluted 1 : 10 and 1 : 100. A loop of each of these suspensions was inoculated on a quadrant of each of the plates and a loop of a freshly prepared suspension of typhoid bacilli on the last quadrant. The number of colonies in each quadrant was recorded after 24 hours' incubation and again after 48 hours' incubation. Only the latter reading is given in the table.

TABLE 2

Results of a Test of Brilliant-Green Culture Media for the Isolation of Typhoid Bacilli

Media		Mixture of Normal Stools			Suspension of Typhoid Bacilli
		1:100	1:10	1:1	
Brilliant-green with sterilized feces	.0003%	42	Numerous	∞	78
	.0005%	27	Numerous	∞	65
	.001 %	0	3	10	60
	.002 %	0	0	9	0
	.004 %	0	1	0	0
Brilliant-green control	.0003%	0	1	4	57
	.0005%	0	1	0	0
	.001 %	0	0	0	0
Eosin + brilliant-green with sterilized feces	.003 %	1	3	50	70
	.005 %	0	0	8	76
	.01 %	0	1	2	69
Eosin + brilliant-green control	.003 %	0	0	6	83
	.005 %	0	0	1	67
	.01 %	0	0	0	40
Plain agar control..............		34	160	∞	85

It is seen from Table 2, first, that the toxicity of brilliant green for the typhoid bacillus is reduced about tenfold by the addition of the eosin; second, that the inhibition of the colon bacilli of the stools is impaired to a much greater degree in the brilliant-green plates than in the eosin brilliant-green plates by the addition of sterilized feces to the medium. Thus, there is practically complete inhibition at 0.0003% brilliant green in the clear agar, while 3 times as much brilliant green (0.001%) is required to produce this result in the agar clouded with sterilized feces. With eosin a given amount of brilliant green produces only slightly less complete inhibition in the clouded

agar than in the clear. A third important fact, which is not brought out in the table, is that the typhoid colonies are quite different in color from the colonies of B. coli on the eosin brilliant-green agar.

The eosin brilliant-green agar containing lactose and saccharose is the medium which we herewith recommend for the isolation of typhoid bacilli from stools. In the article following this[2] we give a number of tables illustrating the mode of action of this plate, from which it is seen that dilutions of the various typhoid stools used which yield numbers of colonies of B. coli on the Endo plate and on the eosin methylene-blue plate of Holt-Harris and Teague[3] usually show only a few colonies besides the typhoid ones on the eosin brilliant-green plate. Time and again typhoid bacilli were recovered from the eosin brilliant-green plate with no difficulty at all when the other two plates, inoculated with the same material, gave negative results. The eosin brilliant-green agar allows the typhoid colonies to develop practically as rapidly as they do on plain nutrient agar, and at the same time shows good inhibition of B. coli. After 18 hours' incubation the typhoid colonies by reflected light are grayish in color, while the colonies of B. coli have red centers. By transmitted light the typhoid colonies are transparent and colorless, while colonies of B. coli show dark centers. On further incubation the typhoid colony becomes pink and the whole colony of B. coli becomes dark-red or dark-purple in color; the typhoid colony remains translucent, while the colony of B. coli becomes more and more opaque. The typhoid colony may show the center somewhat darker than the periphery, but the whole colony is much paler than that of B. coli and is readily distinguished from it. If the colonies are packed closely together on the plate, this differentiation is obscured, the typhoid colonies assuming a darker color and those of B. coli failing to develop centers properly; however, on account of the inhibition which the medium exerts on the growth of most strains of B. coli, this is not likely to occur.

We believe that this medium is more satisfactory for the isolation of typhoid bacilli from stools than any other hitherto recommended.

We prepare the medium as follows: 500 gm. of chopped beef are placed in 1 liter of distilled water and kept in the ice-box over night. The infusion is squeezed through cheese cloth, heated in the Arnold sterilizer, and passed through filter paper. Witte's peptone (1%), chemically pure sodium chlorid (0.5%), and agar (1.5%) are added to the warm infusion, the peptone being first rubbed up into a paste in a little warm water. The flask of medium is

[2] Jour. Infect. Dis., 1916, 18, p. 653.
[3] Ibid., p. 596.

then heated in the autoclave for 30 minutes at 120 C. The reaction is adjusted to +1 by the addition of 2 normal sodium hydrate and then the medium is heated a half hour in the Arnold sterilizer. The medium is again titrated and the reaction is brought to + 1. It is cooled to 55 C., cleared with egg white, and filtered through cotton. It is then placed in flasks in amounts of 100 c.c. or 200 c.c., and heated for 20 minutes in the autoclave at 120 C. It is then stored, ready for use.

In pouring the plates a flask of this agar is melted and its reaction controlled. Lactose (1%) and saccharose (1%) are added. To every 50 c.c. of the agar is added 1 c.c. of a stock 3% solution of yellowish eosin. From the stock 1% brilliant-green solution in 50% alcohol, a ⅙% solution in distilled water is prepared and 1 c.c. of this is added to every 50 c.c. of the agar containing the eosin. After the stains have been distributed uniformly throughout the agar by shaking, plates are poured. These may be inoculated immediately after the agar solidifies, or they may be kept in the ice-box several days and then used.

Until one has become familiar with the appearance of the typhoid and colon-bacillus colonies on this plate and with the general behavior of the medium, control Endo plates or eosin methylene-blue plates should be used. We have recommended 3/50% eosin and 1/300% brilliant green. Agar as prepared in different laboratories possesses somewhat different characteristics and may require slightly more or slightly less brilliant green to yield optimal results. If 1/300% brilliant green does not give good inhibition of B. coli with agar prepared in a certain way, 1/275% brilliant green will probably do so without retarding the growth of B. typhosus. Hence, preliminary tests should be made with typhoid stools, or, if these are not available, with a freshly isolated strain of B. typhosus which has not been in contact with any stains, to determine the optimal amount of brilliant green, the eosin being kept constant in amount; thereafter the agar should always be prepared in the same way from similar materials and this amount of brilliant green used.

As we have not obtained good results on substituting Liebig's meat extract for the meat infusion, we warn against the use of the extract. By employing a meat infusion rendered sugar-free by incubation with B. coli, we have obtained beautiful differentiation of the colonies and excellent inhibition of B. coli, but the typhoid colonies were very small after 24 hours' incubation, and were somewhat reduced in number. After 48 hours' incubation this plate gives good results. On account of the greater trouble in preparing this medium and of the delay in obtaining results, the nutrient agar first described is to be preferred.

A comparative study of this medium, the Endo medium, and the eosin methylene-blue medium is presented in the succeeding article.

A METHOD OF PRESERVING TYPHOID STOOLS FOR DELAYED EXAMINATION AND A COMPARATIVE STUDY OF THE EFFICACY OF EOSIN BRILLIANT-GREEN AGAR, EOSIN METHYLENE-BLUE AGAR, AND ENDO AGAR FOR THE ISOLATION OF TYPHOID BACILLI FROM STOOLS *

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Most laboratories in which examinations of typhoid stools are carried out, receive their specimens at various lengths of time after passage by the patient. In warm weather, stools, particularly if they are liquid, are apt to show, after 12 or more hours, a marked increase in the number of other bacteria as compared to that of the typhoid bacilli present. Since there is no satisfactory enrichment fluid for the typhoid bacillus, the success or failure in isolating typhoid bacilli from stools by our present methods depends primarily on the proportion of typhoid to other bacteria present; Ficker and Hofmann,[1] for example, estimated that 1 : 300 represents the lowest proportion of typhoid that still gives positive results. Hence, it seems highly desirable to preserve the original proportion of typhoid to other bacteria (that is, to prevent overgrowth of the typhoid by the latter) from the time that the stool is passed until it reaches the bacteriologist. The method to be described accomplishes this result, not only for periods of 8, 12, or 24 hours, but for a number of days; it therefore not only obviates the inaccuracies of diagnosis resulting from the usual delay in the arrival of specimens at the laboratory, but also permits of specimens' being sent from a whole state or country to a central station for examination.

Our first experiments in this direction were concerned with the use of hypertonic solutions of sodium chlorid. Preliminary tests as to the effect of the salt solution in various strengths on pure cultures of typhoid bacilli indicated that a 5% solution was the strongest that could be used without causing a rapid drop in the number of typhoid bacilli present. Sodium chlorid in this strength undoubtedly exerts

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[1] Arch. f. Hyg., 1904, 49, p. 229.

a marked inhibitory action on the growth of Bacillus coli. A series of typhoid stools were treated in this way, 5 c.c. of a suspension of each of the stools having been added to 5 c.c. of 10% sodium chlorid and another 5 c.c. of the stools to 5 c.c. of 0.6% sodium chlorid, as control. Attempts were made to isolate typhoid bacilli from both mixtures after they had stood at room temperature for 24 hours, 48 hours, 3 days, and 4 days respectively. In every instance typhoid bacilli could be recovered from the mixture containing 5% sodium chlorid after a longer interval than from the control mixture. Since we later found in glycerin a better medium for this purpose, it is unnecessary to tabulate these earlier results. Table 1 demonstrates the superiority of 25% glycerin over 5% sodium chlorid as a preservative of typhoid stools for delayed examination.

TABLE 1

Comparison of 25% Glycerin and 5% and 0.6% Sodium Chlorid as Preservatives for Typhoid Bacilli in Stools

Period of Delay and Preservative Used	Methylene-blue Eosin Plates							
	1:100,000*		1:10,000		1:1000		1:100	
	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
At once...............	..	..	0	1	0	0	1	0
24 hr. .6% NaCl................	..	..	0	2	1	5	4	36
5% NaCl................	..	..	..	..	0	1	0	1
25% NaCl................	..	..	..	..	0	1	1	1
48 hr. .6% NaCl................	..	..	0	50	0	122	0	∞
5% NaCl................	..	..	..	..	0	2	0	5
25% glycerin..............	..	..	..	..	0	0	0	0
72 hr. .6% NaCl................	0	27	0	100	0	Num.	0	∞
5% NaCl............... ...	..	..	..	..	0	13	0	16
25% glycerin..............	..	..	..	..	0	0	0	0
4 da. .6% NaCl................	0	70	0	Num.	0	∞	0	∞
5% NaCl................	..	..	..	..	0	21	0	85
25% glycerin..............	..	..	..	..	0	0	0	7
5 da. .6% NaCl................	0	Num.	0	Num.	0	∞	0	∞
5% NaCl................	..	..	0	5	0	31	0	Num.
25% glycerin..............	..	..	.	..	0	47	0	25

Num. = numerous; ∞ = infinite numbers.
* Representing the dilution of the suspension of feces used for inoculation.

A stool of a typhoid-carrier, M. B., was rubbed up in 0.6% salt solution and filtered first through cotton and then through filter paper. After this treatment it could be assumed that the typhoid bacilli were uniformly distributed throughout the filtrate. Five cubic centimeters of the filtrate were added to 5 c.c. of 50% glycerin, another 5 c.c. to 10% sodium-chlorid solution, and finally 5 c.c. of the filtrate were added to 5 c.c. of 0.6% sodium chlorid as a control. The test tubes containing these mixtures were kept at room temperature in a dark closet. After intervals of 1, 2, 3, 4, and 5 days dilutions

of the 3 mixtures were prepared and 1 loop of each dilution was spread over one quadrant of an eosin methylene-blue plate and also on a quadrant of an eosin brilliant-green plate.

The number of typhoid colonies and the number of other colonies developing in each quadrant are recorded in the table. The dilution of the suspension of feces with which the quadrant was inoculated is indicated at the top of the column. For the identification of the typhoid bacilli, the appearance of the colonies and a macroscopic agglutination with immune serum on a slide were considered adequate since the stool was obtained from a person known to be a typhoid-carrier.

The eosin methylene-blue plate was recently devised by Holt-Harris and Teague[2] and the eosin brilliant-green plate by Teague and Clurman.[3] The same material was inoculated on both kinds of media in

TABLE 1—*Continued*

COMPARISON OF 25% GLYCERIN AND 5% AND 0.6% SODIUM CHLORID AS PRESERVATIVES FOR TYPHOID BACILLI IN STOOLS

Methylene-blue Eosin Plates				Brilliant-green Eosin Plates							
1:10		1:1		1:1000		1:100		1:10		1:1	
Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
3	6	..	..	0	0	1	0	3	0	35	0
15	Num.	..	..	0	0	9	0	30	20	..	..
4	4	..	..	0	0	2	0	3	0	15	0
1	5	..	..	0	0	0	0	1	0	35	0
0	∞	..	..	0	110	0	Num.	0	∞	0	∞
1	Num.	2	∞	0	0	0	0	1	7	0	80
1	9	22	28	0	0	0	0	0	0	26	2
..	..	..	..	0	Num.	0	Num.	0	∞	..	..
0	Num.	0	∞	0	3	0	26	0	Num.	0	Num.
2	1	18	7	0	0	0	0	0	0	15	1
..	..	..	..	0	28	0	116	..	..	..	..
0	Num.	0	∞	0	7	0	57	0	Num.	0	∞
0	0	12	0	0	0	0	0	1	0	18	0
..	..	..	..	0	Num.	0	∞	..	..	..	..
0	∞	..	..	0	0	0	3	0	Num.	2	∞
2	5	12	73	0	0	1	0	2	0	16	0

order to obtain a more accurate picture of the action of the preservatives. With the eosin brilliant-green plate B. coli is inhibited to a great extent, so that one cannot judge from this plate alone of the relative numbers of typhoid bacilli and colon bacilli present at different intervals. The methylene-blue eosin plate indicates with fair accuracy

[2] Jour. Infect. Dis., 1916, 18, p. 596.
[3] Ibid., p. 647.

the proportion of typhoid bacilli to other bacteria present, but is not able to demonstrate the presence of a very small proportion of typhoid bacilli as does the eosin brilliant-green plate. The inoculation of the two kinds of media with the same material served the additional purpose of allowing us to make a comparative study of the mode of action of these two media.

It is seen from Table 1 that during the first 24 hours both the typhoid and the colon bacilli (we shall call the fecal bacteria colon

TABLE 2

Results of the Use of Various Percentages of Glycerin as Preservative in the Examination of Typhoid Stools

Period of Delay and Percentage of Glycerin Used as a Preservative	Methylene-blue Eosin Plates					
	1:100,000*		1:10,000		1:1000	
	Typhoid	Others	Typhoid	Others	Typhoid	Others
At once...........................	......	......	......	......	4	9
24 hr. .6% NaCl.......	......	......	0	0	4	56
20% glycerin...................	......	......	0	0	4	0
25% glycerin...................	......	......	0	1	5	1
30% glycerin...................	......	......	1	0	3	2
48 hr. .6% NaCl....................	0	10	0	54	0	Num.
20% glycerin...................	......	......	0	1	0	5
25% glycerin....	......	......	0	0	0	3
30% glycerin.........	......	......	0	0	1	2
3 da. .6% NaCl....................	0	28	0	62	0	Num.
20% glycerin...................	......	......	......	......	4	1
25% glycerin...................	......	......	......	......	0	3
30% glycerin...................	......	......	......	......	2	0
4 da. .6% NaCl	0	21	0	63	0	Num.
20% glycerin...................	......	......	......	......	1	2
25% glycerin...................	......	......	......	......	2	2
30% glycerin.................	......	......	......	......	1	1

* Representing the dilution of the suspension of feces used for inoculation.
Num.= numerous; ∞ = infinite numbers.

bacilli, since no attempt was made to separate them into groups in these experiments) increase in number in 0.6% NaCl solution, the latter more rapidly than the former, however. In 5% NaCl solution and 25% glycerin both types of organisms remain constant as to number and proportion.

After 48 hours B. coli has increased enormously in 0.6% NaCl solution, so that typhoid bacilli can no longer be recovered even on the eosin brilliant-green plate. In 5% sodium-chlorid solution B. coli has increased, but to a much less extent than in the control mixture, while the typhoid bacilli have decreased in number. In 25% glycerin there is still very little change in the proportions or numbers of B. coli and typhoid bacilli present.

After 3 days there is a still further increase in B. coli in 5% sodium-chlorid solution and typhoid bacilli are no longer found. In the glycerin mixture B. coli has not increased and typhoid bacilli are reduced to about half the original number. After 4 days the same picture is presented.

After 5 days typhoid bacilli are still present in almost 50% of the original number in 25% glycerin, while colon bacilli have increased only slightly.

TABLE 2—*Continued*

RESULTS OF THE USE OF VARIOUS PERCENTAGES OF GLYCERIN AS PRESERVATIVE IN THE EXAMINATION OF TYPHOID STOOLS

Methylene-blue Eosin Plates		Brilliant-green Eosin Plates							
1:100		1:10,000		1:1000		1:100		1:10	
Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
14	27	......	......	3	7	12	25	Num.	.....
6	Num.	0	2	1	12	4	76	10	Num.
17	24	......	......	0	0	12	2	Num.	.....
39	15	......	......	2	1	20	4	115	35
20	20	......	......	0	0	3	14	Num.	Num.
Few	Num.	......	......	3	10	2	58	4	Num.
13	26	......	......	2	1	15	0	68	30
10	23	......	......	2	0	8	1	95	30
7	5	......	......	0	0	6	2	50	50
0	∞	1	29	0	Num.	0	Num.	0	∞
15	15	......	......	0	0	13	5	51	11
12	16	......	......	0	0	8	1	65	15
3	17	......	......	0	0	5	3	40	15
0	∞	0	38	0	Num.	0	∞	......	.....
10	15	......	......	1	0	6	0	79	8
7	11	......	......	......	......	2	0	67	2
4	9	......	......	0	0	3	0	27	0

Obviously, it will be impossible in practice to obtain constantly the same final percentage of glycerin with different specimens, since stools vary greatly as to the amount of water contained. Hence it was necessary to determine whether fairly wide variations in the percentage of glycerin would still yield satisfactory results.

For this purpose the stool of a typhoid patient (Z. S.) was suspended in 0.6% sodium-chlorid solution and filtered first through cotton and then through filter paper. Five cubic centimeters of filtrate were added to 5 c.c. of 40%, 50%, and 60% glycerin, severally, and to 0.6% sodium-chlorid solution as a control. The test tubes containing the mixtures were kept in the dark at room temperature. At 24-hour intervals dilutions in salt solution were prepared and 1 loop of each was spread over one quadrant of an eosin methylene-blue plate and on a quadrant of an eosin brilliant-green plate. The results of this experiment are recorded in Table 2.

TABLE 3

Results of the Use of Different Percentages of Glycerin as Preservative for Typhoid Stools

Period of Delay and Percentage of Glycerin Used as Preservative	Methylene-blue Eosin Plates							
	1:100,000*		1:10,000		1:1000		1:100	
	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
Patient O. E. X-25. At once	.....	...	...	...	0	0	0	0
24 hr. .6% NaCl	.....	...	)	6	1	49	1	Num.
20% glycerin	.....	...	0	0	0	0	4	2
25% glycerin	.....	...	0	0	0	0	0	1
30% glycerin	.....	...	0	0	0	0	1	0
48 hr. .6% NaCl	0	12	0	41	1	Num.	—	∞
20% glycerin	.....	...	.....	...	0	0	3	16
25% glycerin	.....	...	.....	...	0	0	1	0
30% glycerin	.....	...	.....	...	0	0	2	0
3 da. .6% NaCl	0	3	0	30	—	Num.	—	∞
20% glycerin	.....	...	.....	...	0	32	0	11
25% glycerin	.....	...	.....	...	0	Num.	1	Num.
30% glycerin	.....	...	.....	...	6	4	0	20
4 da. .6% NaCl	0	Num.	0	Num.	0	Num.	—	∞
20% glycerin	....	...	.....	...	1	11	1	Num.
25% glycerin	.....	...	.....	...	0	0	0	5
30% glycerin	.....	...	.....	...	0	0	0	1
5 da. .6% NaCl	0	Num.	—	Num.	—	∞	.....	...
20% glycerin	.....	...	.....	...	0	Num.	5	∞
25% glycerin	.....	...	.....	...	.....	...	0	Num.
30% glycerin	.....	...	.....	...	.....	...	0	1
7 da. .6% NaCl	1	Num.	—	∞	—	∞	.....	...
20% glycerin	.....	...	.....	...	—	Num.	1	∞
25% glycerin	.....	...	.....	...	—	Num.	—	Num.
30% glycerin	.....	...	.....	...	0	0	0	1
9 da. .6% NaCl	0	Num.	—	Num.	—	∞	.....	...
20% glycerin	.....	...	.....	...	0	Num.	0	Num.
25% glycerin	.....	...	.....	...	0	Num.	0	Num.
30% glycerin	.....	...	.....	...	0	0	0	9
Patient M. W. X-25 At once	.....	...	.....	...	0	68	—	Num.
24 hr. .6% NaCl	0	3	0	21	—	Num.	—	∞
20% glycerin	.....	...	0	11	0	75	—	Num.
25% glycerin	.....	...	0	10	0	60	1	Num.
30% glycerin	.....	...	0	10	0	34	1	Num.
48 hr. .6% NaCl	0	30	0	99	—	Num.	—	∞
20% glycerin	.....	...	0	12	0	57	—	Num.
25% glycerin	.....	...	0	8	0	68	1	Num.
30% glycerin	. ...	...	0	6	0	24	—	Num.
3 da. .6% NaCl	0	90	—	Num.	.....	...	.....	...
20% glycerin	0	3	0	28	—	Num.	—	Num.
25% glycerin	0	1	0	9	0	42	—	Num.
30% glycerin	0	0	0	2	1	21	—	Num.
4 da. .6% NaCl	0	34	—	Num.	—	∞	.....	...
20% glycerin	0	0	0	13	0	76	—	Num.
25% glycerin	0	0	0	7	0	57	—	Num.
30% glycerin	0	9	0	4	0	12	—	Num.
5 da. .6% NaCl	—	Num.	—	Num.	—	∞	.....	...
20% glycerin	0	6	0	62	—	Num.	—	∞
25% glycerin	0	18	0	35	—	Num.	—	∞
30% glycerin	0	4	0	11	0	56	—	Num.
9 da. .6% NaCl	—	Num.	—	∞	—	∞	.....	...
20% glycerin	—	5	—	11	—	Num.	—	∞
25% glycerin	0	1	0	15	—·	Num.	—	∞
30% glycerin	0	0	0	2	0	25	—	Num.

TABLE 3—*Continued*

RESULTS OF THE USE OF DIFFERENT PERCENTAGES OF GLYCERIN AS PRESERVATIVE FOR TYPHOID STOOLS

Methylene-blue Eosin Plates				Brilliant-green Eosin Plates							
1:10		1:1		1:1000		1:100		1:10		1:1	
Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
10	15	—	160	0	0	4	0	12	0	86	7
—	∞	. ....	...	1	1	10	12	—	Num.	—	Num.
10	22	.....	...	0	0	3	0	9	0	87	6
12	2	.....	...	0	0	0	0	3	0	46	3
7	7	.....	...	0	0	1	0	9	0	28	1
.....	...	.....	...	0	40	—	Num.	—	Num.	—	∞
11	69	—	Num.	0	0	2	1	17	10	Num.	Num.
2	12	29	44	0	0	0	0	2	3	25	7
2	3	20	31	0	0	0	0	4	0	30	2
.....	...	. ....	...	1	51	—	Num.	—	∞	.....	....
21	Num.	—	Num.	0	6	3	1	17	2	Num.	Num.
3	Num.	21	Num.	0	0	0	0	3	0	15	1
3	Num.	7	Num.	0	0	0	0	1	0	8	0
.....	...	.....	...	0	53	—	Num.	.....	...	.....	....
0	Num.	3	Num.	0	0	1	2	10	4	Num.	Num.
2	Num.	7	Num.	0	0	0	0	0	0	3	1
0	1	13	14	0	0	1	0	0	0	8	0
.....	...	.....	...	0	Num.	—	∞	.....	...	.....	....
—	∞	—	∞	. ....	...	1	10	7	76	—	Num.
2	Num.	4	∞	.....	...	0	0	0	0	5	9
2	7	27	17	.....	...	0	0	0	0	9	1
.....	...	.....	...	0	47	.....	...	.....	...	.....	....
—	∞	—	∞	0	2	2	15	—	88	—	Num.
—	∞	—	∞	0	0	0	0	0	1	5	4
0	0	6	Num.	0	0	0	0	0	0	11	0
.....	...	.....	...	0	34	.....	...	.....	...	.....	....
—	∞	—	∞	0	0	0	0	0	12	0	35
—	∞	—	∞	0	0	0	0	0	0	0	2
5	9	—	Num.	0	0	0	0	0	0	2	0
—	∞	—	∞	0	0	0	0	12	2	91	9
.....	...	.....	...	0	0	0	0	0	7	7	Num.
—	∞	.....	...	0	0	2	0	10	2	Num.	—
—	∞	.....	...	0	0	2	1	9	31	Num.	—
—	∞	.....	...	0	0	0	1	5	9	Num.	—
.....	...	.....	...	0	Num.	2	∞	—	∞	.....	....
—	∞	.....	...	0	0	1	1	8	15	Num.	—
—	∞	.....	...	0	3	1	3	12	17	Num.	—
—	∞	.....	...	0	0	1	1	3	17	Num.	—
.....	...	.....	...	0	0	0	Num.	0	Num.	.....	....
.....	...	.....	...	0	0	1	0	17	1	61	2
.....	...	.....	...	0	0	2	0	5	1	68	7
.....	...	.....	...	0	0	0	1	5	1	42	2
.....	...	.....	...	0	∞	1	∞	6	∞	—	∞
....	...	.....	...	0	0	2	0	5	0	56	13
.....	...	.....	...	0	0	2	0	8	1	62	3
.....	...	. ....	...	0	0	1	0	3	0	59	0
.....	...	.....	...	1	∞	4	∞	—	∞	.....	....
.....	...	.....	...	2	1	3	0	38	3	Num.	20
.....	...	.....	...	0	0	1	0	25	5	Num.	—
.....	...	.....	...	0	0	1	0	11	1	Num.	10
.....	...	.....	...	—	∞	—	∞	...	...	.....	....
.....	...	.....	...	0	0	0	0	12	0	Num.	—
.....	...	.....	...	0	0	0	1	12	2	45	3
.....	...	.....	...	0	0	0	0	2	1	40	6

TABLE 3—*Continued*

RESULTS OF THE USE OF DIFFERENT PERCENTAGES OF GLYCERIN AS PRESERVATIVE FOR TYPHOID STOOLS

Period of Delay and Percentage of Glycerin Used as Preservative	Methylene-blue Eosin Plates							
	1:100,000*		1:10,000		1:1000		1:100	
	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
Patient M. B. **X-25** At once	.....	...	.....	...	0	1	—	Num.
24 hr. .6% NaCl	.....	...	0	6	0	30	1	Num.
20% glycerin	.....	...	.....	...	0	0	0	8
25% glycerin	.....	...	.....	...	0	0	0	2
30% glycerin	.....	...	.....	...	0	0	0	4
48 hr. .6% NaCl	.....	...	0	35	0	110	—	Num.
20% glycerin	.....	...	.....	...	0	25	—	Num.
25% glycerin	.....	...	.....	...	0	0	0	13
30% glycerin	.....	...	.....	...	0	5	0	19
3 da. .6% NaCl	0	13	0	28	—	Num.	—	∞
20% glycerin	.....	...	0	Num.	0	Num.	0	Num.
25% glycerin	.....	...	.....	...	0	Num.	0	Num.
30% glycerin	.....	...	.....	...	0	0	0	Few
4 da. .6% NaCl	0	30	0	50	—	Num.	—	∞
20% glycerin	.....	...	0	2	0	Num.	0	Num.
25% glycerin	.....	...	.....	...	0	0	0	6
30% glycerin	.....	...	.....	...	0	0	0	0
5 da. .6% NaCl	—	Num.	—	Num.	—	∞	—	∞
20% glycerin	.....	...	.....	...	.....	...	0	Num.
25% glycerin	.....	...	.....	...	.....	...	0	Num.
30% glycerin	.....	...	.....	...	.....	...	0	10
7 da. .6% NaCl	0	63	—	Num.	—	∞	.....	...
20% glycerin	.....	...	.....	...	0	Num.	0	∞
25% glycerin	.....	...	.....	...	0	Num.	0	Num.
30% glycerin	.....	...	.....	...	0	0	0	6
9 da. .6% NaCl	—	Num.	—	∞	—	∞	.....	...
20% glycerin	.....	...	.....	...	0	Num.	0	Num.
25% glycerin	.....	...	.....	...	0	Num.	0	Num.
30% glycerin	.....	...	.....	...	0	4	0	6

* Representing the dilution of the suspension of feces used for inoculation.
Num. = numerous; — = too many colonies to make the determination of typhoid bacilli possible; ∞ = infinite numbers.

Table 2 indicates that final concentrations of 20% and of 30% glycerin preserve the typhoid bacilli in the stool and prevent the growth of colon bacilli for a period of 4 days as satisfactorily as 25% glycerin.

Table 3 shows the results of similar experiments with other stools. The feces were filtered through a loose layer of cotton only, in order to approximate the actual conditions of practice more nearly, and the examinations covered longer periods. A dash in the table indicates that the colonies in that quadrant were so numerous and consequently so small that it was impossible to determine whether or not typhoid bacilli were present or, if present, it was impossible to estimate their number accurately.

TABLE 3—*Continued*

Results of the Use of Different Percentages of Glycerin as Preservative for Typhoid Stools

Methylene-blue Eosin Plates				Brilliant-green Eosin Plates							
1:10		1:1		1:1000		1:100		1:10		1:1	
Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
2	Num.	5	Num.	1	0	0	0	0	0	6	2
—	∞	.....	...	0	1	1	7	25	52	—	Num.
—	Num.	6	Num.	0	0	0	0	1	0	8	2
4	Num.	6	Num.	0	0	0	0	1	1	16	1
—	Num.	6	Num.	0	0	0	0	6	0	4	1
—	∞	.....	...	0	22	—	Num.	—	∞	—	∞
1	Num.	4	∞	0	1	0	0	1	2	4	74
0	Num.	2	Num.	0	0	0	0	0	0	2	2
—	Num.	—	Num.	0	0	0	0	2	0	4	1
.....	...	.....	...	2	14	—	Num.	—	∞	.....	....
0	Num.	.....	...	0	0	0	0	0	0	6	10
0	Num.	4	Num.	0	0	0	0	0	0	0	1
0	Num.	2	Num.	0	0	0	0	0	0	2	0
.....	...	.....	...	0	6	0	31	—	Num.	—	∞
—	Num.	.....	...	0	0	0	0	1	0	6	20
0	Num.	1	Num.	0	0	0	0	0	0	2	1
1	Num.	8	Num.	0	0	0	0	0	0	3	1
.....	...	.....	...	—	Num.	—	∞	—	∞	—	∞
2	Num.	5	∞	.....	...	0	0	0	4	7	47
0	∞	5	∞	.....	...	0	0	0	0	5	1
1	Num.	8	Num.	.....	...	0	0	0	0	14	4
.....	...	.....	...	0	Num.	—	∞	.....	...	.....	....
3	∞	2	∞	0	0	0	1	0	17	—	Num.
0	∞	2	∞	0	0	0	1	0	2	8	0
0	Num.	1	Nun	0	0	0	0	0	0	9	10
.....	...	.....	...	—	Num.	.....	...	.....	...	.....	....
0	Num.	0	∞	0	0	0	1	1	1	11	15
0	∞.	4	∞	0	0	0	0	0	0	5	1
0	Num.	2	Num	0	0	0	0	0	0	3	0

It is seen from Table 3 that B. coli is not inhibited so well in 20% glycerin as in the other two mixtures, and typhoid bacilli decrease in number a little more rapidly in 30% glycerin than in the others, but all three percentages yield eminently satisfactory results. By furnishing 30% glycerin in sterile bottles to which some of the stool is to be added it will be easy to obtain in practice final concentrations lying between 20% and 30%.

In order to determine more accurately the total number of viable organisms in the glycerin mixture after different intervals of time it was decided to plate in plain nutrient agar 0.5 c.c. of appropriate dilutions and record the number of colonies.

A mixture of 6 typhoid stools and 6 normal stools was used to insure a great variety of fecal bacteria. The stools were emulsified in salt solution, thoroughly mixed together, and filtered through a thin layer of cotton. Five

TABLE 4

RESULTS OF THE USE OF GLYCERIN AS A PRESERVATIVE IN THE EXAMINATION OF TYPHOID STOOLS

Period of Delay and Percentage of Glycerin Used as a Preservative	Number of Colonies per 0.5 c.c.	Endo Plates							
		1:10,000,000*		1:1,000,000		1:100,000		1:10,000	
		Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
At once..................	2,000,000	.....	.....	.....	.....	.....	.....	.....	.....
24 hr. { 25% glycerin.....	1,800,000	.....	.....	.....	.....	.....	.....	0	8
.6% NaCl.......	65,000,000	.....	.....	.....	.....	0	Num.	0	Num.
48 hr. { 25% glycerin.....	1,500,000	.....	.....	.....	.....	.....	.....	0	3
.6% NaCl.......	758,000,000	.....	.....	.....	.....	0	Num.	0	Num.
3 da. { 25% glycerin....	800,000	.....	.....	0	26	0	Num.	—	∞
.6% NaCl.......	900,000,000	.....	.....						
4 da. { 25% glycerin.....	350,000	.....	.....	0	Num.	—	∞	—	∞
.6% NaCl........	15,000,000,000	.....	.....						
7 da. { 25% glycerin.....	....	.....	.....	.....	.....	.....	.....	.....	.....
.6% NaCl........	....	—	∞	—	∞	.....	.....	.....	.....

* Representing the dilution of the suspension of feces used for inoculation.

Num. = numerous; ∞ = infinite numbers; — = too many colonies to make and accurate determination of typhoid bacilli possible.

TABLE 4—Continued

RESULTS OF THE USE OF GLYCERIN AS A PRESERVATIVE IN THE EXAMINATION OF TYPHOID STOOLS

Period of Delay and Percentage of Glycerin Used as a Preservative	Number of Colonies per 0.5 c.c.	Methylene-blue Eosin Plates					
		1:10,000		1:1000		1:100	
		Typhoid	Others	Typhoid	Others	Typhoid	Others
At once.............	2,000,000	.....	.....	0	14	2	96
24 hr. { 25% glycerin...................	1,800,000	0	0	1	10	0	65
.6% NaCl.....................	65,000,000	0	35	0	Num.	—	∞
48 hr. { 25% glycerin...................	1,500,000	0	1	0	6	1	36
.6% NaCl.....................	758,000,000	0	Num.	—	∞	—	∞
3 da. { 25% glycerin...................	800,000	.....	.....	0	3	0	31
.6% NaCl.....................	900,000,000	—	∞	—	∞	.....	.....
4 da. { 25% glycerin...................	350,000	.....	.....	0	1	1	25
.6% NaCl.....................	15,000,000,000	—	∞	—	∞	.....	.....
7 da. { 25% glycerin...................	....	.....	.....	0	2	0	4
.6% NaCl.....................	....	—	∞	—	∞	.....	.....

Num. = numerous; ∞ = infinite numbers; — = too many colonies to make and accurate determination of typhoid bacilli possible.

TABLE 4—*Continued*

RESULTS OF THE USE OF GLYCERIN AS A PRESERVATIVE IN THE EXAMINATION OF TYPHOID STOOLS

Endo Plates								Methylene-blue Eosin Plates					
1:1000		1:100		1:10		1:1		1:10,000,000		1:1,000,000		1:100,000	
Ty-phoid	Others	Ty-phoid	Others	Ty-phod	Others	Ty-phoid	Others	Ty-phoid	Others	Ty-phoid	Others	Ty-phoid	Others
2	24	0	140	—	Num.	.....	.....	.....	.....	.....	.....	.....	.....
1	Num.	0	Num.	—	Num.	.....	.....	.....	.....	.....	.....	.....	.....
0	Num.	—	∞	.....	.....	.....	.....	.....	.....	.....	.....	0	3
0	6	0	74	—	Num.	.....	.....	.....	.....	.....	.....	.....	.....
--	Num.	—	∞	.....	.....	.....	.....	.....	.....	.....	.....	0	54
0	10	1	51	—	Num.	—	∞	.....	.....	.....	.....	.....	.....
—	∞	....	.....	.....	.....	.....	.....	.....	.....	0	14	0	Num.
0	7	0	45	0	Num.	—	∞	.....	.....	.....	.....	.....	.....
—	∞	.....	.....	.....	....	.....	.....	.....	.....	0	15	0	Num.
0	2	0	26	—	Num.	—	Num.	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	—	Num.	—	∞	—	∞

TABLE 4—*Continued*

RESULTS OF THE USE OF GLYCERIN AS A PRESERVATIVE IN THE EXAMINATION OF TYPHOID STOOLS

Methylene-blue Eosin Plates				Brilliant-green Plates									
1:10		1:1		1:10,000		1:1000		1:100		1:10		1:1	
Ty-phoid	Others	Ty-phoid	Others	Ty-phoid	Others	Ty-phoid	Others	Ty-phoid	Others	Ty-phoid	Others	Ty-phoid	Others
—	Num.	.....	.....	.....	.....	0	0	5	0	21	6	Num.	—
—	Num.	.....	.....	.....	.....	0	0	2	0	11	7	Num.	—
.....	.....	.....	.....	0	1	0	56	0	Num.	—	∞	.....	.....
—	Num.	.....	.....	.....	.....	0	0	0	0	6	2	Num.	—
.....	... .	.....	.....	0	12	0	48	—	Num.	—	∞	.....	.....
4	Num.	—	∞	.....	.....	.....	.....	.....	.....	6	7	90 (2 loops)	25
.....	.....	.....	.....	0	9	—	Num.	—	∞	.....	.....	.....	.....
—	Num.	—	∞	.....	.....	.....	.....	.....	.....	5	1	Num. (2 loops)	—
.....	.....	.....	.....	0	Num.	—	Num.	—	∞	.....	.....	.....	.....
0	Num.	—	∞	.....	.....	.....	.....	.....	.....	10	10	Num.	—
.. ..	.....	.....	...	—	Num.	—	∞	.....	.....	.....	.....	.....	.....

cubic centimeters of the filtrate were added to 5 c.c. of 50% glycerin in 0.6% sodium-chlorid solution and another 5 c.c. to 5 c.c. of 0.6% sodium-chlorid solution alone. Dilutions were prepared at intervals and 1 loop of each was inoculated on a quadrant of an Endo, an eosin methylene-blue, and an eosin brilliant-green plate. On the 3rd, 4th, and 7th days 2 loops instead of 1 were inoculated on the eosin brilliant-green plate. At the same time 0.5 c.c. amounts of each of appropriate dilutions of the glycerin mixture and of the 0.6% sodium-chlorid-solution control were plated in ordinary nutrient agar. The results of this experiment are recorded in Table 4.

It is seen in Table 4 that in the glycerin mixture there was a gradual decrease in the number of viable bacteria from day to day, so that after 3 days less than half the original number were present; in the con-

TABLE 5

Results of the Use of Glycerin plus Broth as a Preservative in the Examination of Typhoid Stools

Period of Delay and Percentage of Glycerin Used as Preservative	Endo Plates			
	1:1000*		1:100	
	Typhoid	Others	Typhoid	Others
Patient A. P. VIII-4 At once...	4	23	10	80
24 hr. { 25% glycerin and 4% NaCl..............	2	8	1	24
20% glycerin..............................	0	16	4	86
25% glycerin..............................	0	5	10	30
25% glycerin and 4% NaCl in broth....	0	1	0	35
25% glycerin in broth..................	1	2	8	42
48 hr. { 25% glycerin and 4% NaCl..............	0	1	5	13
20% glycerin..............................	0	8	4	101
25% glycerin..............................	1	0	17	12
25% glycerin and 4% NaCl in broth....	0	1	4	23
25% glycerin in broth..................	0	4	4	39
3 da. { 25% glycerin and 4% NaCl..............	0	0	2	16
20% glycerin..............................	0	41	—	Num.
25% glycerin..............................	0	2	4	8
25% glycerin and 4% NaCl in broth....	0	5	3	11
25% glycerin in broth..................	0	21	0	45
5 da. { 25% glycerin and 4% NaCl..............	0	5	0	29
20% glycerin..............................	—	Num.	—	∞
25% glycerin..............................	1	0	1	2
25% glycerin and 4% NaCl in broth....	0	9	2	13
25% glycerin in broth..................	1	11	12	33

* Representing dilution of the suspension of feces used for inoculation.
Num. = numerous; ∞ = infinite numbers; — = too many colonies to make an accurate determination of the typhoid bacilli.

trol mixture the bacteria increased from 2 million to 65 million during the first 24 hours and the typhoid bacilli were during this short interval so overgrown by the other bacteria that they were not recovered from any of the plates. From the glycerin mixture typhoid bacilli were readily recovered on the 7th day.

Altho the proportion of typhoid to colon bacilli in 25% glycerin remains constant or is altered in favor of the typhoid bacilli, yet there is a gradual decrease in the absolute number of typhoid bacilli present. It was thought that this decrease might perhaps be obviated by adding a small amount of broth to the glycerin.

The stool of a typhoid patient (A. P.) was rubbed up in salt solution and filtered through a thin layer of cotton. Fifty-percent glycerin was prepared in ordinary nutrient broth and 5 c.c. of this were mixed with 5 c.c. of the filtered feces. This mixture was compared with 20% and 25% glycerin in 0.6% NaCl solution and with 25% glycerin in combination with 4% sodium-chlorid solution. The results are shown in Table 5.

TABLE   5—*Continued*

RESULTS OF THE USE OF GLYCERIN PLUS BROTH AS A PRESERVATIVE IN THE EXAMINATION OF TYPHOID STOOLS

Endo Plates		Brilliant-green Eosin Plates					
1:10		1:1000		1:100		1:10	
Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
—	Num.	0	0	9	12	81	6
—	Num.	1	0	6	1	38	2
—	Num.	3	0	18	3	Num.	—
—	Num.	1	0	18	0	Num.	—
—	∞	1	0	8	0	34	3
—	Num.	ṽ	0	7	1	57	8
—	Num.	0	0	2	0	21	1
—	Num.	0	0	0	4	50	31
—	Num.	0	0	6	0	80	0
—	Num.	0	0	0	0	20	0
—	Num.	0	0	3	0	58	2
—	Num.	0	0	1	0	3	0
—	∞	0	3	2	7	Few	Num.
—	Num.	0	0	3	0	32	0
—	Num.	1	0	1	0	12	0
—	Num.	2	0	3	2	48	2
6	57	0	0	0	0	17	0
—	∞	0	5	0	60	—	Num.
20	36	1	0	3	0	19	0
11	67	0	0	2	0	21	0
—	Num.	1	0	7	0	47	0

There seems to be no practical advantage gained by adding sodium chlorid or broth to 25% glycerin. Table 5 shows that there is better inhibition of B. coli in 25% glycerin than in 20% and that the addition of broth to 25% glycerin impairs the inhibition of B. coli slightly.

In the previous experiments, all of which show that typhoid bacilli can be recovered from glycerin mixtures long after it is impossible to obtain them from the salt-solution control, the stools were carefully

TABLE 6

RESULTS OF THE USE OF GLYCERIN AS PRESERVATIVE IN ROUTINE EXAMINATION OF
TYPHOID STOOLS

Period of Delay and Percentage of Glycerin Used as a Preservative	Methylene-blue Eosin Plates					
	1:10,000		1:1000		1:100	
	Typhoid	Others	Typhoid	Others	Typhoid	Others
Patient E. O. X-4 At once......	......	......	......	......	......	......
24 hr. 30% glycerin......	......	......	......	......	......	......
48 hr. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
3 da. { 30% glycerin......	1	8	6	15	—	Num.
{ Control stool......	4	115	—	Num.	—	x
7 da. { 30% glycerin......	0	4	1	Num.	4	Num.
{ Control stool......	0	Num.	—	Num.	—	x
10 da. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
15 da. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
Patient M. W. X-4 At once......	......	......	......	......	......	......
48 hr. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
10 da. { 30% glycerin......	0	Num.	0	Num.	0	Num.
{ Control stool......	0	Num.	0	Num.	—	x
15 da. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
Patient J. L. X-4 At once......	......	......	......	......	......	......
48 hr. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
3 da. 30% glycerin......	4	4	12	17	—	95
7 da. { 20% glycerin......	1	7	0	7	5	31
{ Control stool......	0	Num.	—	Num.	—	∞
10 da. 30% glycerin......	......	......	0	2	0	24
15 da. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
22 da. 30% glycerin......	......	......	0	Num.	—	∞
Patient M. B. X-4 At once......	......	......	......	......	......	......
48 hr. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......
3 da. 30% glycerin......	3	56	8	Num.	—	∞
7 da. { 30% glycerin......	0	22	5	21	4	Num.
{ Control stool......	0	∞	—	∞	—	∞
10 da. { 30% glycerin......	0	4	0	10	0	Num.
{ Control stool......	......	......	......	......	......	......
15 da. { 30% glycerin......	......	......	......	......	......	......
{ Control stool......	......	......	......	......	......	......

TABLE 6—*Continued*

RESULTS OF THE USE OF GLYCERIN AS PRESERVATIVE IN ROUTINE EXAMINATION OF
TYPHOID STOOLS

Methylene-blue Eosin Plates		Brilliant-green Eosin Plates							
1:10		1:1000		1:100		1:10		1:1	
Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
......	......	0	0	1	0	17	7	Num.	—
......	......	......	......	0	2	1	15	—	Nuu.
......	......	0	0	0	1	2	5	12	Num.
......	......	0	1	0	1	0	33	0	Num.
—	Num.	......	......	......	......	......	......	......	.....
—	∞	......	......	......	......	......	......	......	.....
1	Num.	0	2	6	19	9	Num.	—	∞
—	∞	0	Num.	0	Num.	—	∞	—	∞
......	......	0	0	0	0	0	0	0	0
......	......	0	30	0	Num.	—	∞	—	∞
......	......	0	0	0	0	0	0	0	2
......	......	0	63	0	Num.	—	∞	—	∞
......	......	0	0	0	1	10	7	Num.	—
......	......	0	0	0	2	0	S	0	Num.
......	.. ...	0	0	0	1	2	2	7	11
—	∞	0	0	0	0	0	0	6	5
—	∞	0	0	0	0	0	Num.	—	Num.
......	......	0	5	0	33	0	Num	—	∞
......	......	0	Num.	—	Num.	—	∞	—	∞
......	......	1	0	5	0	28	6	Num.	—
......	......	0	0	1	0	1	0	Num.	—
......	......	0	2	3	7	0	22	—	Num.
—	Num.	......	......	......	......	......	......	......	.....
3	Num.	4	0	14	4	Num.	Num.	—	∞
—	∞	0	Num.	—	∞	—	∞	—	∞
2	60	0	0	0	0	0	1	13	2
......	......	0	0	0	1	0	2	2	28
......	......	0	23	0	Num.	—	∞	—	∞
—	∞	......	......	0	1	0	1	0	3
......	......	0	4	0	15	0	Num.	—	∞
......	......	1	0	0	15	0	Num.	—	∞
......	......	0	3	0	Num.	0	Num.	—	∞
—	∞	......	......	......	......	......	......	......	.....
—	∞	4	9	5	21	25	Num.	—	∞
—	∞	0	Num.	—	∞	—	∞	—	∞
—	∞	0	0	0	1	3	3	8	32
......	......	0	2	0	15	0	Num.	—	∞
......	......	1	1	0	0	0	5	7	13
......	......	0	15	0	Num.	—	Num.	—	∞

TABLE 6—*Continued*

RESULTS OF THE USE OF GLYCERIN AS PRESERVATIVE IN ROUTINE EXAMINATION OF
TYPHOID STOOLS

Period of Delay and Percentage of Glycerin Used as a Preservative	Methylene-blue Eosin Plates					
	1:10,000		1:1000		1:100	
	Typhoid	Others	Typhoid	Others	Typhoid	Others
Patient A. M. X-4 At once...	......	......	......	......	......	......
24 hr. { 30% glycerin...	......	......	......	......	......	......
{ Control stool...	......	......	......	......	......	......
48 hr. { 30% glycerin...	......	......	......	......	......	......
{ Control stool...	......	......	......	......	......	......
3 da. { 30% glycerin...	10	0	—	Num.	—	Num.
{ Control stool...	5	Num.	1	∞	—	∞
10 da. { 30% glycerin...	......	......	......	......	......	......
{ Control stool...	......	......	......	......	......	......
15 da. { 30% glycerin...	......	......	......	......	......	......
{ Control stool...	......	......	......	......	......	......
22 da. 30% glycerin...	......	......	2	Num.	8	Num.
Patient E. O. X-11 At once...	......	......	......	......	......	......
24 hr. { 30% glycerin...	0	1	2	0	3	8
{ Control stool...	0	0	3	1	8	10
48 hr. { 30% glycerin...	0	0	0	0	5	0
{ Control stool...	0	1	0	0	0	10
4 da. { 30% glycerin...	1	11	—	Num.	—	Num.
{ Control stool...	1	0	—	Num.	—	Num.
6 da. { 30% glycerin...	0	0	0	0	0	0
{ Control stool...	0	6	0	0	0	0
15 da. 30% glycerin...	......	......	0	Num.	—	∞
Patient M. B. X-11 At once...	......	......	......	......	......	......
24 hr. { 30% glycerin...	10	16	14	81	Num.	—
{ Control stool...	1	7	2	68	—	Num.
48 hr. { 30% glycerin...	2	14	6	20	—	Num.
{ Control stool...	0	6	1	45	8	Num.
Patient M. W. X-11 At once...	......	......	......	......	......	......
24 hr. { 30% glycerin...	4	8	8	22	35	85
{ Control stool...	0	65	0	Num.	—	∞
48 hr. { 30% glycerin...	2	1	1	1	12	21
{ Control stool...	0	Num.	0	Num.	—	∞
6 da. { 30% glycerin...	......	......	......	......	......	......
{ Control stool...	......	......	......	......	......	......
15 da. 30% glycerin...	......	......	......	......	......	......
Patient A. M. X-11 At once...	......	......	......	......	......	......
24 hr. { 30% glycerin...	4	24	1	84	2	Num.
{ Control stool...	0	60	0	Num.	—	∞
48 hr. { 30% glycerin...	0	Num.	0	Num.	—	Num.
{ Control stool...	0	Num.	0	Num.	—	∞
4 da. { 30% glycerin...	0	Num.	0	Num.	—	Num.
{ Control stool...	0	Num.	0	—	—	∞

TABLE 6—*Continued*

RESULTS OF THE USE OF GLYCERIN AS PRESERVATIVE IN ROUTINE EXAMINATION OF TYPHOID STOOLS

Methylene-blue Eosin Plates		Brilliant-green Eosin Plates							
1:10		1:1000		1:100		1:10		1:1	
Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others	Typhoid	Others
......	......	0	0	0	0	0	2	8	10
......	......	0	0	0	0	3	8	—	Num.
......	......	0	0	1	0	10	3	29	2
......	......	0	0	0	0	0	2	25	35
......	......	0	30	0	Num.	—	∞	—	∞
—	Num.	0	0	0	0	0	3	15	Num.
—	∞	0	Num.	—	∞	—	∞	—	∞
......	......	0	0	1	0	2	0	28	0
......	......	0	11	2	53	12	Num.	—	∞
......	......	0	0	0	0	6	2	42	8
......	......	0	1	0	4	0	45	0	Num.
10	0	......	......	......	......	......	......	......	......
......	......	......	......	2	0	26	0	Num.	—
40	80	0	0	4	0	13	0	Num.	0
36	41	0	0	4	0	42	0	Num.	0
24	0	0	0	0	0	3	0	26	0
......	......	0	0	2	0	24	0	......	......
......	......	......	......	......	......	......	......	......	......
......	......	......	......	......	......	......	......	......	......
4	1	0	0	1	0	3	0	37	0
3	0	0	0	0	0	0	0	2	1
—	∞	......	......	0	0	0	3	0	Num.
......	......	......	......	2	0	51	0	Num.	1
—	∞	25	0	60	0	Num.	0	∞	—
—	∞	4	0	28	0	—	Num.	Num.	—
—	Num.	0	0	10	0	Num.	—	∞	2
—	∞	0	0	0	0	17	0	Num.	0
......	......	......	......	1	0	2	0	Num.	—
Num.	—	4	0	33	0	Num.	0	∞	0
—	∞	0	1	0	12	0	65	0	Num.
—	Num.	0	0	1	0	Num.	—	Num.	—
......	......	0	5	0	18	0	Num.	......	......
......	......	0	0	7	9	50	33	∞	—
......	......	0	34	0	66	0	Num.	—	∞
......	......	......	......	0	0	3	0	25	7
......	......	......	......	0	0	1	29	0	Num.
—	∞	7	0	33	0	Num.	0	Num.	Few
—	∞	0	0	0	1	2	9	0	Num.
—	∞	0	0	0	0	1	4	0	32
......	......	0	2	0	1	0	1	......	......
......	......	......	......	......	......	......	......	......	......
......	......	......	......	......	......	......	......	......	......

rubbed up in salt solution and filtered through cotton in order to remove the coarser particles. Such treatment of the stools would be impracticable in routine hospital work. We have therefore preserved a series of stools in the following manner.

Wide-mouthed, screw-topped bottles, such as are ordinarily used for sputum, were sterilized and filled about two-thirds full with 30% glycerin in 0.6% sodium-chlorid solution. The typhoid stool was added until the bottle was nearly full and a portion of the same stool was placed in a sterile empty bottle to serve as a control. If the stool was fluid, the glycerin mixture was merely shaken after the top had been screwed on the bottle; if the stool was solid, it was crushed in the 30% glycerin with a wooden spatula or glass rod, no attempt being made to break up thoroughly all the small lumps. At intervals of one to several days the glycerin mixture was shaken well, dilutions were prepared, and plates were inoculated as in the preceding experiments. At the same time a bit of the control stool was emulsified in salt solution to make a suspension of approximately the same density as that of the glycerin mixture. This suspension was diluted and inoculated on plates in a similar manner. Examples of stools treated in this way are shown in Table 6.

Table 6 shows that the proportion of typhoid to colon bacilli is not decreased in 30% glycerin for a period of a week or more, while there is an increase of B. coli in the control stool at the expense of the typhoid. Thus similar results are obtained when the stools are merely crushed in the 30% glycerin, and when they are thoroughly emulsified and the coarser particles removed by filtration through cotton.

When specimens are to be sent through the mail or by express, the feces should be added to 30% glycerin, not more than 1 part of feces to 2 parts of glycerin, in a bottle with a screw top such as is used for sputum. If the stool is solid, it should be broken up with a spatula or splinter of wood. The top to the bottle should be screwed down tightly and a bit of adhesive plaster wrapped around it to prevent its working loose. The bottle should then be shaken well, to distribute the stool material throughout the glycerin, and packed in double containers such as are used for mailing cultures. The 30% glycerin is prepared by adding glycerin to sterile 0.6% sodium-chlorid solution.

It is believed that the method just described will afford material aid in the stamping out of typhoid fever by making it easier to discover typhoid-carriers. The method should also assist the practicing physician in ascertaining, before he dismisses his typhoid-convalescent patients, whether or not they are still discharging typhoid bacilli with their stools.

With regard to the comparison of the three kinds of media used for the isolation of typhoid bacilli in these experiments, little need be said, since the facts are sufficiently obvious in the tables. It is seen that with almost every stool used large numbers of colonies of B. coli appear on the Endo and eosin methylene-blue plates, when the same material yields very few colon colonies on the eosin brilliant-green agar; it is seen that the typhoid organisms grow up as well on the latter medium as on the other two, the same material yielding practically the same number of typhoid colonies on the three different plates when only a few B. coli are present; it is seen finally that typhoid bacilli are time and again recovered on the eosin brilliant-green plate from material that yields negative results on the other two, while in no instance do the latter plates show typhoid colonies when the eosin brilliant-green plate is negative. It therefore follows that the eosin brilliant-green plate, if properly prepared, is far superior to the other two for the isolation of typhoid bacilli from stools.

The Endo and eosin methylene-blue plates yield similar results (see Table 4), but the typhoid colonies are more readily found on the latter, which is therefore to be preferred.

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